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(54) Title: <b>REGULATION OF bcl-2 GENE EXPRESSION</b>  (57) Abstract  The present invention provides novel anticodon oligomers and methods of using them for controlling the growth of cancer cells expressing the bcl-2 gene.		

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REGULATION OF bcl-2 GENE EXPRESSIONReference to Government Grants

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Field of the Invention

The present invention relates to the field of treatments for cancer and more particularly to the field of anticod oligomer treatments for cancer.

Related Application Data

This application is a continuation-in-part of Serial No. 07/840,716 filed February 21, 1992, which was a continuation in part of Serial No. 07,288,692 filed December 22, 1988, which has been abandoned.

Background of the Invention

Current approaches to cancer treatment suffer from a lack of specificity. The majority of drugs that have been developed are natural products or derivatives that either block enzyme pathways or randomly interact with DNA. Due to low therapeutic indices, most cancer treatment drugs are accompanied by serious dose-limiting toxicities. The administration of drugs to treat cancer kills not only cancer cells but also normal non-cancerous cells. Because of these deleterious effects, treatments that are more specific for cancerous cells are needed.

It has been found that a class of genes, the oncogenes, plays a large role in the transformation and maintenance of the cancerous state and that turning off these genes, or otherwise inhibiting their effects, can return a cell to a normal phenotype. The role of oncogenes in the etiology of many human cancers has been

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reviewed in Bishop, "Cellular Oncogenes and Retroviruses," *Science*, 235:305-311 (1987). In many types of human tumors, including lymphomas and leukemias, the human bcl-2 gene is overexpressed, and may be associated with tumorigenicity (Tsujimoto et al. Involvement of the bcl-2 gene in human follicular lymphoma, *Science* 228:1440-1443 (1985)).

Antisense oligodeoxynucleotides are one example of a specific therapeutic tool with the potential for ablating oncogene function. These short (usually about 30 bases) single-stranded synthetic DNAs have a complementary base sequence to the target mRNA and form a hybrid duplex by hydrogen bonded base pairing. This hybridization can be expected to prevent expression of the target mRNA code into its protein product and thus preclude subsequent effects of the protein product. Because the mRNA sequence expressed by the gene is termed the sense sequence, the complementary sequence is termed the antisense sequence. Under some circumstances, inhibition of mRNA would be more efficient than inhibition of an enzyme's active site, since one mRNA molecule gives rise to multiple protein copies.

Synthetic oligodeoxynucleotides complementary to (antisense) mRNA of the c-myc oncogene have been used to specifically inhibit production of c-myc protein, thus arresting the growth of human leukemic cells in vitro, Holt et al., *Mol. Cell Biol.* 8:963-973 (1988), and Wickstrom et al., *Proc. Natl. Acad. Sci. USA*, 85:1028-1-32 (1988). Oligodeoxynucleotides have also been employed as specific inhibitors of retroviruses, including the human immunodeficiency virus (HIV-I), Zamecnik and Stephenson, *Proc. Natl. Acad. Sci. USA*, 75:280-284 (1978) and Zamecnik et al., *Proc. Natl. Acad. Sci. USA*, 83:4143-4146 (1986).



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Summary of the Invention

The invention provides anticode oligomers and methods for inhibiting growth of cancer cells. The growth of lymphoma or leukemia cells, which are types of lymphocytes, are inhibited by the anticode oligomers and methods of the invention. An anticode oligomer complementary to at least an effective portion of the mRNA sense strand to the human *bcl-2* gene is provided and cells are then contacted with the anticode oligomer in a concentration sufficient to inhibit growth of the cells. The methods of the invention are suitable for inhibiting growth of lymphoma/leukemia cells that express the human *bcl-2* gene and have a t (14; 18) chromosomal translocation as well as those that express the *bcl-2* gene but do not have a t (14; 18) chromosomal translocation.

In accordance with preferred embodiments, the anticode oligomer is substantially complementary to a strategic site in the pre-mRNA sense strand or substantially complementary to the mRNA. A preferred strategic site is the translation-initiation site of the pre-mRNA coding strand. Alternative strategic sites include coding sites for splicing, transport or degradation. The subject anticode oligomer either in its "native," unmodified form -- oligonucleotide -- or as a derivative, is brought into contact with the target lymphoma or leukemia cells. For *in vivo* therapeutic use, a derivative of the "native" oligonucleotide, such as the phosphorothioate form is preferable since it is believed that these forms are more resistant to degradation, notwithstanding the fact that response times to some analogues, such as the phosphorothioate analogs, has been found to be somewhat slower than to the "native" form of the oligonucleotide.

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A preferred anticode oligomer, denominated herein the TI-AS (translation initiation anticode oligomer) is an oligodeoxynucleotide which straddles the translation-initiation site of the mRNA coding strand of the human *bcl-2* gene and is complementary to this region. More preferably, this nucleotide comprises a TAC portion which is complementary to the ATG initiation sequence of the coding strand for the *bcl-2* gene, and preferably further comprises flanking portions of two to about one hundred bases, more preferably from about five to about twenty bases, which are complementary to portions of the *bcl-2* gene coding strand flanking said initiation sequence. The TI-AS nucleotide has been found effective at inhibiting the growth of the target cells both in the presence and absence of serum.

Alternatively, the anticode oligomer comprises an antisense nucleotide complementary to at least an effective portion of the splice donor site of the pre-mRNA coding strand for the human *bcl-2* gene. More particularly, this nucleotide comprises a CA portion which is complementary to the GT splice donor of the *bcl-2*, and again comprises flanking portions of two to about one hundred bases, preferably from about five to about twenty bases, which are complementary to portions of the *bcl-2* gene coding strand flanking said splice donor.

In yet another embodiment, the anticode oligomer is complementary to at least an effective portion of the splice acceptor region of the pre-mRNA coding strand for the human *bcl-2* gene. This oligomer comprises at least a TC portion which is complementary to the AG splice acceptor of the *bcl-2* gene, and again comprises flanking portions of two to about one hundred, preferably from about five to about twenty bases which are complementary to portions of the *bcl-2* gene coding

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strand flanking said acceptor. The subject oligomer may also be selected to overlap the coding site for the 26 kDa protein, *bcl-2*-alpha or for the 22 kDa protein, *bcl-2*-beta, protein products of the *bcl-2* gene.

5 Preferably the oligomer is selected to minimize homology with anticode oligomers for pre-mRNA or mRNA coding strands for other gene sequences.

Accordingly, a primary object of the present invention is the provision of novel anticode oligomers, 10 which are useful in inhibiting the growth of cancer cells. The present invention also includes compositions for inhibiting the growth of tumor cells, which compositions comprise the anticode oligomer of the present invention together with a pharmaceutically 15 acceptable carrier.

A further object of the present invention is the provision of methods for inhibiting the growth of cancer cells using said anticode oligomers. As a feature of the present invention, it was discovered that average 20 reductions of 30-40% in the relative levels of *bcl-2* protein markedly enhanced the sensitivity of lymphoma cells, in particular, t(14;18)-containing lymphoma cell lines to cancer chemotherapeutic agents, including conventional anticancer drugs. Such reductions were 25 achieved by introducing into tumor cells an anticode oligomer which binds to either pre-mRNA or mRNA expressed from the *bcl-2* gene. Two methods were used in the present invention to introduce said anticode oligomers to tumor cells. One method involved contacting the tumor 30 cells with a composition comprising the anticode oligomers. Another method involved transfecting the tumor cells with a vector encoding an antisense oligonucleotide. Introducing an anticode oligomer to tumor cells achieved a reduction of *bcl-2* expression and

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increases the chemosensitivity of neoplastic cells to cancer chemotherapeutic agents or anticancer drugs.

Accordingly, the present invention achieved a method of killing tumor cells by introducing to tumor cells anticode oligomers which reduce *bcl-2* gene expression or impair Bcl-2 protein function before contacting the cells with cancer chemotherapeutic agents. The cancer chemotherapeutic agents reduced the numbers of viable malignant cells, and the portion of tumor cells killed was greater than the portion which would have been killed by the same amount of drug in the absence of introducing the anticode oligomer oligodeoxynucleotide to the cells.

These and other objects of the present invention will become apparent from the following detailed description.

#### Brief Description of the Drawings

Figures 1 (a - d) show graphs of the effects of varying concentrations of antisense oligodeoxynucleotides on inhibition of cell proliferation.

Figures 2(a) and (b) show graphs of the concentration dependence of inhibition of cell proliferation by antisense normal and phosphorothioate oligodeoxynucleotides. Oligodeoxynucleotide additions to cultures included TI-AS phosphorothioate (o and o; two separate experiments), TI-S phosphorothioate ( $\Delta$ ), TI-AS normal ( $\square$ ), and TI-S normal ( $\Delta$ ).

Figure 3 shows the results of gel electrophoresis of six antisense oligonucleotides targeted against the translation initiation site of *bcl-2* mRNA.

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Figures 4(a) and (b) show the degree of DNA fragmentation resulting from oligonucleotide treatment of RS11846 cells. Figure 4(a) shows the effect of oligonucleotides targeted against the translation initiation site. Figure 4(b) shows the effect of oligonucleotides directed against the 5'-cap region of *bcl-2* mRNA.

Figure 5 is a graph showing the concentration-dependence of inhibition by an antisense oligonucleotide targeted against the translation initiation site of *bcl-2* mRNA.

Figures 6(a) and (b) are graphs showing the results of immunofluorescence analysis of *bcl-2* protein levels in oligonucleotide-treated cells.

Figures 7(a-d) are FACS profiles for 697 cells before and after treatment with *bcl-2* antisense oligonucleotides.

Figure 8 (a-c) show *bcl-2* antisense oligodeoxynucleotides producing sequence-specific reductions in *bcl-2* mRNA and *bcl-2* protein and producing increased sensitivity of SU-DHL-4 cells to cancer chemotherapeutic drugs.

Figures 9(a) and (b) demonstrate the differential effects of *bcl-2* antisense oligomers on chemosensitivity of 32D-*bcl-2* and 32D-BHRF-1 cells.

Figures 10 (a-b) show reduction of chemoresistance of RS11846 cells from inducible *bcl-2* antisense expression from an expression plasmid.

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Figure 11 shows methylphosphonate/phosphodiester bcl-2 antisense oligomers inducing death of DOHH2 lymphoma cells.

5 Figure 12 shows methylphosphonate (MP)/Phosphodiester (PO) chimeric oligomers inhibiting growth of MCF-7 human breast cancer cells.

Figures 13(a-c)) show optimization of antisense bcl-2 oligomer sequences.

#### Detailed Description of the Invention

10 According to the invention, anticode oligomers are provided for inhibiting cancer cell growth, for increasing the sensitivity of cancer cells to cancer chemotherapeutic agents, or for inducing cancer cell death alone or in combination with any one or more cancer  
15 chemotherapeutic agents.

#### Definitions

As used herein, the term "anticode oligomers" means anticode oligonucleotides and analogs thereof and refers to a range of chemical species that recognize  
20 polynucleotide target sequences through hydrogen bonding interactions with the nucleotide bases of the target sequences. The target sequences may be single- or double-stranded RNA or single- or double-stranded DNA.

The anticode oligonucleotides and analogs  
25 thereof may be RNA or DNA, or analogs of RNA or DNA, commonly referred to as antisense oligomers or antisense oligonucleotides. Such RNA or DNA analogs comprise but are not limited to 2'-O-alkyl sugar modifications, methylphosphonate, phosphorothioate, phosphordithioate,  
30 formacetal, 3'-thioformacetal, sulfone, sulfamate, and nitroxide backbone modifications, and analogs wherein the base moieties have been modified. In addition, analogs

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of oligomers may be polymers in which the sugar moiety has been modified or replaced by another suitable moiety, resulting in polymers which include, but are not limited to, morpholino analogs and peptide nucleic acid (PNA) analogs (Egholm, et al. Peptide Nucleic Acids (PNA) - Oligonucleotide Analogues with an Achiral Peptide Backbone, (1992)).

Anticodon analogs may also be mixtures of any of the oligonucleotide analog types together or in combination with native DNA or RNA. At the same time, the oligonucleotides and analogs thereof may be used alone or in combination with one or more additional oligonucleotides or analogs thereof. The oligonucleotides may be from about 10 to about 1,000 nucleotides long. Although oligonucleotides of 10 to 100 nucleotides are useful in the invention, preferred oligonucleotides range from about 15 to about 24 bases in length.

Anticodon oligonucleotides and analogs thereof also comprise conjugates of the oligonucleotides and analogs thereof. (John Goodchild, Conjugates of Oligonucleotides and Modified Oligonucleotides: A Review of Their Synthesis and Properties, Bioconjugate Chemistry, Volume 1 No. 3, May/June (1990)). Such conjugates having properties to improve the uptake, pharmacokinetics, and nuclease resistance of the oligonucleotide, or the ability to enhance cross-linking or cleavage of the target sequence by the oligonucleotide.

As used herein, the term "cell proliferation" refers to cell division rate/cell cycle. The term "growth," as used herein, encompasses both increased cell numbers due to faster cell division and due to slower rates of cell death.

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As used herein, *bcl-2* gene expression refers to *bcl-2* protein production from the human *bcl-2* gene; e.g. reduced *bcl-2* gene expression means reduced levels of *bcl-2* protein.

5           As used herein, "strategic sites" are defined as any site which when bound by the claimed anticodon molecules or analogs thereof results in inhibiting expression of the *bcl-2* gene.

10           As used herein, the term "sequence portion" is a portion of the nucleotide sequence of an RNA oligonucleotide. In appropriate contexts, "sequence portion" may refer to a portion of the nucleotide sequence of a DNA segment or DNA oligonucleotide.

15           Uncontrolled cell proliferation is a marker for a cancerous or abnormal cell type. Normal, non-cancerous cells divide regularly, at a frequency characteristic for the particular type of cell. When a cell has been transformed into a cancerous state, the cell divides and proliferates uncontrollably. Inhibition of proliferation modulates the uncontrolled division of the cell.  
20           Containment of cell division often correlates with a return to a non-cancerous state.

25           A human gene termed *bcl-2* (B cell lymphoma/leukemia-2) is implicated in the etiology of some common lymphoid tumors, Croce et al., "Molecular Basis Of Human B and T Cell Neoplasia," in: *Advance in Viral Oncology*, 7:35-51, G. Klein (ed.), New York: Raven Press, 1987. High levels of expression of the human *bcl-2* gene have been found in all lymphomas with t (14;  
30           18) chromosomal translocations including most follicular B cell lymphomas and many large cell non-Hodgkin's lymphomas. High levels of expression of the *bcl-2* gene have also been found in certain leukemias that do not



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have a t(14; 18) chromosomal translocation, including most cases of chronic lymphocytic leukemia acute, many lymphocytic leukemias of the pre-B cell type, neuroblastomas, nasopharyngeal carcinomas, and many adenocarcinomas of the prostate, breast, and colon.

(Reed et al., Differential expression of bcl-2 protooncogene in neuroblastoma and other human tumor cell lines of neural origin. Cancer Res. 51:6529 (1991); Yunis et al. Bcl-2 and other genomic alterations in the prognosis of large-cell lymphomas. New England J. Med. 320:1047; Campos et al. High expression of bcl-2 protein in acute myeloid leukemia is associated with poor response to chemotherapy. Blood 81:3091-3096 (1993); McDonnell et al. Expression of the protooncogene bcl-2 and its association with emergence of androgen-independent prostate cancer. Cancer Res. 52:6940-6944 (1992); Lu Q-L, et al. Bcl-2 protooncogene expression in Epstein Barr Virus-Associated Nasopharyngeal Carcinoma, Int. J Cancer 53:29-35 (1993); Bonner et al. bcl-2 protooncogene and the gastrointestinal mucosal epithelial tumor progression model as related to proposed morphologic and molecular sequences, Lab Invest. 68:43A (1993)).

While not limited to the following explanation, the present invention exploits cellular mechanisms concerned with normal cell death. Because most types of cells have a finite life span and are programmed to die, uncontrollable cell accumulation can also result because of a defect in normal cell death mechanisms rather than through an increased rate of cell division. The bcl-2 gene contributes to the pathogenesis of cancer primarily by prolonging cell survival rather than accelerating cell division.

Antisense oligomers suitable for use in the invention include nucleotide oligomers which are two to

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two hundred nucleotide bases long; more preferably ten to forty bases long; most preferably twenty bases long. The oligonucleotides are preferably selected from those oligonucleotides complementary to strategic sites along the pre-mRNA of *bcl-2*, such as the translation initiation site, donor and splicing sites, or sites for transportation or degradation.

Blocking translation at such strategic sites prevents formation of a functional *bcl-2* gene product. It should be appreciated, however, that any combination or subcombination of anticode oligomers, including oligonucleotides complementary or substantially complementary to the *bcl-2* pre-mRNA or mRNA that inhibit cell proliferation is suitable for use in the invention. For example, oligodeoxynucleotides complementary to sequence portions of contiguous or non-contiguous stretches of the *bcl-2* RNA may inhibit cell proliferation and would thus be suitable for use in the invention.

It should also be appreciated that anticode oligomers suitable for use in the invention may also include oligonucleotides flanking those complementary or substantially complementary to such sequence portions as the strategic or other sites along the *bcl-2* mRNA. The flanking sequence portions are preferably from two to about one hundred bases, more preferably from about five to about twenty bases in length. It is also preferable that the anticode oligomers be complementary to a sequence portion of the pre-mRNA or mRNA that is not commonly found in pre-mRNA or mRNA of other genes to minimize homology of anticode oligomers for pre-mRNA or mRNA coding strands from other genes.

Preferred antisense, or complementary, oligodeoxynucleotides are listed in Table 1.

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TABLE I

*bcl-2* Oligodeoxynucleotides

translation initiation

antisense (TI-AS) 3'...CCCTTCCTACCGCGTGCGAC...5'

5 *bcl-2* 5'...CTTTTCCTCTGGGAAGGATGGCGCACGCTGGGAGA...3'

splice donor

antisense (SD-AS) 31...CCTCCGACCCATCCACGTAG...5'

*bcl-2* 5'...ACGGGGTAC...GGAGGCTGGGTAGGTGCATCTGGT...3'

splice acceptor

10 antisense (SA-AS) 3'...GTTGACGTCCTACGGAAACA...5'

*bcl-2* 5'...CCCCCAACTGCAGGATGCCTTTGTGGAAGTGTACGG...3'

It will be appreciated by those skilled in the art to which this invention pertains, that anticode oligomers having a greater or lesser number of substituent nucleotides, or that extend further along the *bcl-2* mRNA in either the 3' or 5' direction than the preferred embodiments, but which also inhibit cell proliferation are also within the scope of the invention.

It is preferable to use chemically modified derivatives or analogs of anticode oligomers in the performance of the invention rather than "native" or unmodified oligodeoxynucleotides. "Native" oligodeoxynucleotides can be conveniently synthesized with a DNA synthesizer using standard phosphoramidite chemistry. Suitable derivatives, and methods for preparing the derivatives, include phosphorothioate, Stein et al., *Nucl. Acids Res.*, 16:3209-3221 (1988); methylphosphonate, Blake et al., *Biochemistry* 24:6132-6138 (1985) and alphadeoxynucleotides, Morvan et al., *Nucl. Acids Res.* 14:5019-5032 (1986), 2'-O-methyl-ribonucleosides (Monia et al. Evaluation of 2'-modified oligonucleotides containing 2' deoxy gaps as antisense inhibitors of gene expresssion. *J. Biol. Chem.* 268:14514-14522 (1993)), and covalently-linked derivatives such as

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acridine, Asseline et al., *Proc. Natl Acad. Sci. USA* 81:3297-3201 (1984); alkylated (e.g., N-2-chlorocethylamine), Knorre et al., *Biochemie* 67:783-789 (1985) and Vlassov et al., *Nucl. Acids Res.* 14:4065-4076 (1986); phenazine, Knorre et al., *supra*, and Vlassov et al., *supra*; 5-methyl-N<sup>4</sup>-N<sup>6</sup>-ethanocytosine, Webb et al., *Nucl. Acids Res.* 14:7661-7674 (1986); Fe-ethylenediamine tetraacetic acid (EDTA) and analogues, Boutorin et al., *FEBS Letter's* 172:43-46 (1984); 5-glycylamido-1, 10-o-phenanthroline, Chi-Hong et al., *Proc. Natl. Acad. Sci. USA* 83:7147-7151 (1986); and diethylenetriamine-pentaacetic acid (DTPA) derivatives, Chu et al., *Proc. Natl. Acad. Sci.* 82:963-967 (1985). All of the above publications are hereby specifically incorporated by reference as if fully set forth herein.

The anticode oligomer of the present invention can also be combined with a pharmaceutically acceptable carrier for administration to a subject or for ex-vivo administration. Examples of suitable pharmaceutical carriers are a variety of cationic lipids, including, but not limited to N-(1-2,3-dioleoyloxy)propyl)-n,n,n-trimethylammonium chloride (DOTMA) and dioleoylphosphatidylethanolamine (DOPE)]. Liposomes are also suitable carriers for the anticode oligomers of the invention.

The anticode oligomers may be administered to patients by any effective route, including intravenous, intramuscular, intrathecal, intranasal, intraperitoneal, subcutaneous injection, in situ injection and oral administration. Oral administration requires enteric coatings to protect the claimed anticode molecules and analogs thereof from degradation along the gastrointestinal tract. The anticode oligomers may be mixed with an amount of a physiologically acceptable carrier or diluent, such as a saline solution or other

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suitable liquid. The anticode oligomers may also be combined with liposomes or other carrier means to protect the anticode molecules or analogs thereof from degradation until they reach their targets and/or facilitate movement of the anticode molecules or analogs thereof across tissue barriers.

The anticode oligomers may also be useful for ex vivo bone marrow purging. Normally, the amounts of conventional cancer chemotherapeutic agents or drugs and irradiation that a patient can receive are limited by toxicity to the marrow, i.e., anemia (fatigue, heart failure), thrombocytopenia (bleeding), neutropenia (infection). Thus, in order to deliver sufficient concentrations of drugs and irradiation to totally eradicate the tumor, the physician would simultaneously destroy the patient's normal bone marrow cells leading to patient demise. Alternatively, large amounts of bone marrow can be surgically extracted from the patient and stored in vitro while the patient receives aggressive conventional treatment. The patient can then be rescued by reinfusion of their own bone marrow cells, but only if that marrow has been "purged" of residual malignant cells. The claimed anticode oligomers could be used to remove residual malignant cells from the bone marrow.

The anticode oligomers are administered in amounts effective to inhibit cancer or neoplastic cell growth. The actual amount of any particular anticode oligomer administered will depend on factors such as the type of cancer, the toxicity of the anticode oligomer to other cells of the body, its rate of uptake by cancer cells, and the weight and age of the individual to whom the anticode oligomer is administered. Because of inhibitors present in human serum that may interfere with the action of the anticode oligomer an effective amount of the anticode oligomer for each individual may vary.

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An effective dosage for the patient can be ascertained by conventional methods such as incrementally increasing the dosage of the anticode oligomer from an amount ineffective to inhibit cell proliferation to an effective amount. It is expected that concentrations presented to cancer cells in the range of about 0.001 micromolar to about 100 micromolar will be effective to inhibit cell proliferation.

The anticode oligomers are administered to the patient for at least a time sufficient to inhibit proliferation of the cancer cells. The anticode oligomers are preferably administered to patients at a frequency sufficient to maintain the level of anticode oligomers at an effective level in or around the cancer cells. To maintain an effective level, it may be necessary to administer the anticode oligomers several times a day, daily or at less frequent intervals. Anticod oligomers are administered until cancer cells can no longer be detected, or have been reduced in number such that further treatment provides no significant reduction in number, or the cells have been reduced to a number manageable by surgery or other treatments. The length of time that the anticod oligomers are administered will depend on factors such as the rate of uptake of the particular oligodeoxynucleotide by cancer cells and time needed for the cells to respond to the oligodeoxynucleotide. *In vitro*, maximal inhibition of neoplastic cell growth by "native," unmodified anticod oligomers occurred two days after initiation of cultures,, whereas phosphorothioate oligodeoxynucleotides required 4 to 7 days to achieve maximal inhibition. *In vivo*, the time necessary for maximal inhibition of cell proliferation may be shorter or longer.

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The anticode oligomers of the invention may be administered to patients as a combination of two or more different anticode oligomer oligodeoxynucleotide sequences or as a single type of sequence. For instance, TI-AS and SD-AS could be administered to a patient or TI-AS alone.

It is also believed that the anticode oligomers of the invention may be useful in the treatment of autoimmune diseases. Autoimmune diseases are those diseases in which the body's immune system has malfunctioned in some way. Administration of the anticode oligomers of the invention to a person having an autoimmune disease should inhibit proliferation of bcl-2 overexpressing lymphocytes, which would in turn reduce the symptoms of the autoimmune disease. For use in treating autoimmune diseases, the anticode oligomers would be administered as described herein.

### EXAMPLES

#### General Methods

The Examples below use the following protocols:

A. Cells and Cell Cultures. Human leukemic cells lines used for these studies were RS11846 follicular lymphoma cells, 697 pre-B cell acute lymphocytic leukemic cells, and JURAT T cell acute lymphocytic leukemic cells as described in Tsujimoto et al., *Proc. Natl. Acad. Sci. USA*, 83:5214-5218 (1986) and Weiss et al., *Proc. Natl. Acad. Sci. USA*, 138:2169-2174 (1987). Human peripheral blood lymphocytes (PBL) were isolated from fresh whole blood as described in Reed et al., *J. Immunol.*, 134:314-319 (1985). All lymphoid cells were cultured at  $5 \times 10^5$  cells/ml in RPMI medium supplemented with 1 mM glutamine, antibiotics, and either 5-10% (v:v) fetal bovine serum (FBS), 5-10% (v:v) calf serum (CS) (both from Hyclone Laboratories), or 1% (v:v) HLI concentrated supplement (Ventrex Laboratories) for

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serum-free cultures. Murine fibroblast cell lines were added at  $10^3$  cells/cm<sup>2</sup> in DMEM medium containing glutamine, antibiotics and 5-10% (v:v) FCS. Fibroblast cell lines were NIH 3T3 cells, 3T3-B-alpha-S cells, and 3T3-B-alpha-AS cells. These latter two cell lines are NIH 3T3 cells that express high levels of a human *bcl-2*-alpha cDNA in either the sense or antisense orientation, respectively, by virtue of stable transfection with expression vectors constructs.

B. Measurement of Cellular Growth. Growth of cell lines cultured in the presence or absence of anticod oligomers was measured by two methods: cell counts using a hemocytometer; and DNA synthesis by assaying [<sup>3</sup>H]-thymidine incorporation essentially as described in Reed et al., *J. Immunol.*, 134:314-319 (1985). Briefly, cells were cultured in 96-well flat-bottomed microtiter plates (Falcon) at 0.2 ml/well. At appropriate times, cells were resuspended, 25 µl removed from cultures for cell counting, and this volume replaced with 25 µl of 20 UCi/ML [<sup>3</sup>H]-thymidine (specific activity 6.7 Ci/mmol) (New England Nuclear). Microtiter cultures were then returned to 37°C and 95% air: 5% CO<sub>2</sub> atmosphere for 8 hours before lysing cells on glass filters and determining relative levels of [<sup>3</sup>H]-thymidine incorporation into DNA by scintillation counting. Cell counts were performed in the presence of trypan blue dye to determine the concentration of viable cells in duplicate microcultures.

MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] dye reduction assays were performed by the method of Tada, et al. *J. Immunol Methods* 93, 157 (1986), and confirmed to be within the linear range of the assay under the conditions described here. The number of viable cells per well was extrapolated from standard curves that were included with each assay and



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that consisted of serial two-fold dilutions of exponentially growing SU-DHL-4 cells in HL-1 medium, beginning with  $10^6$  cells/ml (0.2ml/well). Samples were assayed in triplicate and the OD600<sub>nm</sub> for a media/reagent blank was subtracted from all values prior to calculations.

C. RNA Blot Analysis. Total cellular RNA was isolated by a guanidinium isothiocyanate/phenol procedure as described in Chomczynski et al., *Analyt. Biochem.*, 162:156-139 (1987). The polyadenylated fraction was purified by oligodeoxythymidine-cellulose chromatography as described in Aviv et al., *Proc. Natl. Acad. Sci. USA*, 69:1408-1412 (1972). Approximately 5  $\mu$ g aliquots of mRNA were size-fractionated in 0.8% agarose/6% formaldehyde gels and transferred to nylon membranes. Blots were prehybridized, hybridized, and washed exactly as described in Reed et al., *Mol. Cell Biol.*, 5:3361-3366 (1985), using either a  $^{32}$ P-cDNA for human *bcl-2*, as described in Tsujimoto et al., *Proc. Natl. Acad. Sci. USA*, 83:5214-5218 (1986), or a murine *bcl-2* probe, pMBCL5.4 as described in Negrini et al., *Cell*, 49:455-463 (1987). Blots were exposed to Kodak XAR film with intensifying screens at -70°C for 1-10 days. Eluting  $^{32}$ P-*bcl-2* probes from membranes and rehybridizing with a  $^{32}$ P probe for mouse beta-2-microglobulin verified nearly equivalent amounts of mRNA for all samples on blots.

#### EXAMPLE 1

##### Preparation of Anticodone oligomers

Normal and phosphorothioate

oligodeoxynucleotides were synthesized using an Applied Biosystems 380B DNA synthesizer, and purified by HPLC reverse-phase chromatography (PRP-1 column) as described in Stein et al., *Nucl. Acids Res.*, 16:3209-3221 (1988) which is specifically incorporated as if fully set forth herein. In some cases it was necessary to further purify

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oligodeoxynucleotides by C18-Sep-Pak chromatography (Waters Associates, Millipore, Inc.), as described previously in Kern et al. , *J. Clin. Invest.*, 81:237-244 (1988), to eliminate nonspecific cytotoxic activity.

5 Oligodeoxynucleotides eluted in 30% acetonitrile were evaporated to dryness, resuspended at 1-2 mM in sterile Dulbecco's phosphate-buffered saline or Hanks' buffered salt solution (both from Gibco), and stored at -80°C in small aliquots.

10 Table 1 shows the oligodeoxynucleotides synthesized and their relation to the sense-strand of the human *bcl-2* gene. Portions of the sequence of the coding strand of the human *bcl-2* gene are shown, including the translation initiation site (top), splice donor site  
15 (middle), splice acceptor region (bottom), and empirically selected sites within the 5' untranslated portion of *bcl-2* pre-mRNA. The ATG initiation codon, GT splice donor, and AG splice acceptor consensus sequences are in boxes.

20 The sequences of the oligodeoxynucleotides synthesized for these investigations are presented, and their relation to human *bcl-2* mRNA is indicated. The TI-AS oligodeoxynucleotide is antisense at the translation initiation site and TI-S is its complementary  
25 sense version. SD-AS and SD-S are oligodeoxynucleotides having antisense and sense orientations, respectively, relative to the splice donor region.

30 The oligodeoxynucleotide TI-AS straddles the predicted translation-initiation site of *bcl-2* mRNAs and is complementary (antisense) to this region. As a control, the sense version of this 20 bp oligodeoxynucleotide, TI-S, was also synthesized.

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In an effort, to specifically block splicing of *bcl-2* mRNAs, a 20 bp antisense oligodeoxynucleotide, SD-AS, was synthesized that overlaps the splice donor site in *bcl-2* primary transcripts. In addition, a complementary sense oligodeoxynucleotide, SD-S, was prepared as depicted in Table 1. The human *bcl-2* gene gives rise to several transcripts through alternative splice site selections, see Tsujimoto et al., *Proc. Natl. Acad. Sci. USA*, 83:5214-5218 (1986). The preponderance of these transcripts depend upon splicing and encode a 26 kDa protein, *bcl-2*-alpha. One minor transcript, however, does not undergo a splice and consequently encodes a 22 kDa protein *bcl-2*-beta. The SD-AS oligodeoxynucleotide can thus potentially block maturation of most but not all *bcl-2* transcripts.

#### EXAMPLE 2

##### Treatment of Serum for In Vitro Investigations of Antisense Normal Oligodeoxynucleotides

Because normal oligodeoxynucleotides are sensitive to degradation by nucleases present in serum, the efficacy of the TI-AS oligodeoxynucleotide in fetal bovine serum (FBS) heated for 30 minutes at 56°C (the usual procedure for inactivating serum complement) was contrasted with the efficacy of TI-AS in FBS heated for 1 hour at 68°C, a temperature sufficient for irreversible inactivation of many nucleases. The RS11846 follicular lymphoma cell line was used. RS11846 cells contain a t(14; 18) chromosomal translocation that deregulates *bcl-2* expression, resulting in the accumulation of high levels of *bcl-2* mRNAs, Tsujimoto et al., *Proc. Natl. Acad. Sci. USA*, 83:5214-5218 (1986).

RS11846 follicular lymphoma cells were cultured in medium containing 5% (vol:vol) fetal bovine serum (FBS) that had been heated at 56°C for 0.5 hours or at 68°C for 1 hour. TI-AS normal oligodeoxynucleotide was

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added at the initiation of culture, and the density of viable cells determined two days later.

The TI-AS normal oligodeoxynucleotide was more effective in 68 C-treated serum at suppressing the growth in culture of these lymphoma cells. In all subsequent experiments, sera heated at 68°C for 1 hour prior to use were used in cultures. This treatment did not impair the growth-supporting capacity of the sera.

### EXAMPLE 3

#### Specific Inhibition of Lymphoid Cell Growth by Antisense Normal Oligodeoxynucleotides

Antisense normal oligodeoxynucleotides directed against the translation initiation site (TI-AS) and the splice donor site (SD-AS) of *bcl-2* transcripts were tested for their ability to suppress the proliferation of normal and neoplastic lymphoid cells.

RS11846 follicular lymphoma cells, JUKRAT T cell leukemia cells, and freshly isolated peripheral blood lymphocytes were cultured in medium containing 10% (vol:vol) FBS that had been heated at 68°C for one hour. various concentrations of normal oligodeoxynucleotides were added at the initiation of culture, including: TI-AS, TI-S, SD-AS, and SD-S. Relative DNA synthesis was measured in cultures after 2-3 days by [<sup>3</sup>H]-thymidine incorporation. Data were calculated as a percentage of control cultures containing volumes of PBS or HBSS equivalent to oligodeoxynucleotide-treated cultures, and represent the mean ( $\pm$  standard deviation) of duplicate cultures.

Similar data were obtained by measuring cell counts, excluding cold thymidine inhibition as an explanation for the suppression of DNA synthesis observed in cultures treated with antisense oligodeoxynucleotides.

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As shown in Figures 1(a), (b), (c) and (d) both the TI-AS and SD-AS oligodeoxynucleotides inhibited the growth of RS11846 cells in a concentration-dependent manner. The SD-AS oligonucleotide was less effective in inhibiting cell growth than the TI-AS oligodeoxynucleotide. In contrast to these antisense oligodeoxynucleotides, sense oligodeoxynucleotides (TI-S and SD-S) were not inhibitory even at concentrations of up to 250  $\mu$ G/ml. Moreover, non-sense oligodeoxynucleotides (i.e., those having the same base composition as the antisense oligodeoxynucleotides but with scrambled sequences) also failed to suppress the proliferation of RS11846 cells. The data thus indicate that antisense oligodeoxynucleotides can specifically block the proliferation of these tumor cells. Several other leukemic cell lines that express the *bcl-2* gene were also tested for inhibition of their proliferation by TI-AS and SD-AS oligonucleotides. As with the JURKAT T cell acute lymphocytic leukemic cells, in every case a specific and concentration-dependent decrease in the growth of these human leukemic cells in cultures containing antisense oligodeoxynucleotides was observed.

It has been demonstrated that *bcl-2* expression is transiently induced in normal human peripheral blood lymphocytes (PBL) when these cells are stimulated to proliferate, suggesting that this gene may play a role in the regulation of normal lymphocyte growth, Reed et al., *Science* 236:1295-1297 (1987). The capacity of antisense oligodeoxynucleotides to impair the growth of PBL cultured with a monoclonal antibody, OKT3 (Van den Elsen et al., *Nature* 312:413-418 (1984)), that stimulates their proliferation was therefore tested. PBL were stimulated with 50  $\mu$ l of purified OKT3 monoclonal antibody. As shown in Figures 1 (a), (b), (c) and (d) the TI-AS oligodeoxynucleotide specifically suppressed the proliferation of PBL in a concentration-dependent manner.

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These antisense normal oligodeoxynucleotides thus suppressed the growth in culture of leukemic cells that constitutively express the *bcl-2* gene and of normal lymphocytes where in *bcl-2* expression is inducible.

5

EXAMPLE 4Time-Course of Inhibition by  
Antisense Normal Oligodeoxynucleotides

The kinetics of inhibition by antisense oligodeoxynucleotides was examined in cultures of RS11846 follicular lymphoma cells and of 697 pre-B cell acute lymphocytic leukemia cells. Both of these neoplastic B cell lines transcribe and accumulate *bcl-2* mRNAs at high levels, Tsujimoto et al., *Proc. Natl. Acad. Sci. USA*, 83:5214-5218 (1986).

15

RS11846 follicular lymphoma and 697 pre-B cell leukemia cells were cultured in medium containing 10% (vol:vol) 68°C-treated FBS and normal oligodeoxynucleotides. Cells were cultured with 50 µg/ml TI-AS, 100 µg/ml SD-AS, 50 µg/ml TI-S (RS11846 cells) or 100 µg/ml SO-S (697 cells), or PBS as a control. DNA synthesis (kcpm/10<sup>5</sup> viable cells) and cell densities (10<sup>5</sup> viable cells/ml) were measured at various times after initiation of cultures.

20

Antisense normal oligodeoxynucleotides markedly inhibited DNA synthesis measured in cultures of these cells within 24 hours. Diminished cell densities were readily apparent in these cultures within 2 days. Antisense normal oligodeoxynucleotides thus rapidly inhibited the *in vitro* growth of leukemic cells. The action of antisense oligodeoxynucleotides was specific, since sense oligodeoxynucleotides did not impair proliferation in these cultures. Though cell viabilities often declined during the later days of culture no increase in cell death was seen during the first 1-2 days

25

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of culture with antisense oligodeoxynucleotides, suggesting a non-cytotoxic mechanism.

#### EXAMPLE 5

##### Comparison of Different Serum Preparations

5           Inhibition of proliferation of leukemic cells with antisense oligodeoxynucleotides can vary greatly depending on the lot of serum used in cultures.

10           To determine the effects of serum of inhibition of proliferation, relative levels of DNA synthesis were measured in cultures of 697 pre-B cell leukemia cells 2 days after addition of 200  $\mu$ M TI-AS normal oligodeoxynucleotide. Cells were cultured in medium supplemented with 1% (vol:vol) HL1-concentrate (serum-free condition), 5% (vol:vol) of two different  
15           lots of calf serum (CS1 and CS2), or 5% (vol:vol) of two different lots of fetal bovine serum (FBS1 and FBS2). All sera were heated at 68°C for 1 hour prior to use in cultures.

20           The normal TI-AS oligodeoxynucleotide markedly inhibited DNA synthesis (92%) and cellular proliferation in serum-free cultures (HL1) of 697 cells. This antisense oligodeoxynucleotide was equally effective (94%) in cultures containing 5% (v:v) of one of the lots of fetal bovine serum (FBS2). In contrast, inhibition  
25           was significantly reduced in cultures containing other serum preparations (CS1, CS2, FBS1). It has been generally observed that antisense normal oligodeoxynucleotides are less effective in cultures supplemented with calf serum (CS) than in those  
30           containing fetal bovine serum (FBS).

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EXAMPLE 6Concentration Dependence of Inhibition by Antisense  
Normal Oligodeoxynucleotides in Serum-Free Cultures

697 pre-B cell leukemia cells were cultured in medium with either 1% (vol:vol) HL1-concentrate (serum-free conditions or 5% (vol:vol) 68°C-treated FBS2). Relative levels of DNA synthesis and cellular densities measured after 2 days in cultures containing various concentrations of normal TI-AS oligodeoxynucleotide.

The TI-AS oligodeoxynucleotide was inhibitory at lower concentrations when used in serum-free cultures. At 100  $\mu$ M, for instance, no inhibition of cellular proliferation was seen in FBS2-containing cultures, whereas cell counts were reduced by approximately 75% in serum-free cultures. At higher concentrations of antisense oligodeoxynucleotides (200-250  $\mu$ M), however, inhibition of 697 cellular proliferation was comparable in both types of cultures. The increased efficacy of normal oligodeoxynucleotides in serum-free cultures was specific, since the sense oligonucleotide (TI-S) was not inhibitory at the same concentrations.

EXAMPLE 7Antisense Phosphorothioate Oligodeoxynucleotides:Time Course of Inhibition

To contrast the efficacy of phosphorothioate oligodeoxynucleotides with that of normal oligodeoxynucleotides with regard to inhibition of human leukemic cell growth, phosphorothioate oligodeoxynucleotides were cultured with 697 pre-B cell leukemia cells and the effects on inhibition were measured. 697 pre-B cell leukemia cells were cultured in serum-free medium for various times before measuring DNA synthesis (kcpm) and cell densities ( $10^6$  cells/ml). Cells were seeded at an initial density of either  $0.2 \times 10^5$



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cells/ml or  $0.5 \times 10^5$  cells/ml. Culture conditions were 25  $\mu$ M TI-AS phosphorathioate, 25  $\mu$ M TI-S phosphorothioate, and control cultures treated with HBSS.

To avoid experimental variation due to differences among lots of sera, 697 leukemic cells were cultured in serum-free conditions. When cultured at an initial seeding density of  $0.5 \times 10^6$  cells/ml, 697 cells achieved maximal DNA synthesis and cellular densities at 4-5 days. Addition of 25  $\mu$ M sense phosphorothioate oligodeoxynucleotide (TI-S) at the initiation of these cultures had little effect on 697 cell growth. In replicate cultures containing 25  $\mu$ M antisense phosphorothioate (TI-AS), however, some diminution in DNA synthesis was evident within 2 days and was maximal at 4-5 days. Maximal inhibition of 697 cell growth, as determined by cell counts, was seen at 6 days after initiation of cultures.

When 697 cells were initially seeded at  $0.2 \times 10^6$  cells/ml, the antisense phosphorothioate oligodeoxynucleotide, TI-AS, resulted in only slight inhibition at 2 days, attaining maximal suppression of DNA synthesis in these cultures at day 7. As with normal oligodeoxynucleotides, this inhibition by phosphorothioate oligodeoxynucleotides appeared to be mediated through non-cytotoxic mechanisms, since cellular viabilities did not decline until late in the course of culture. Compared with normal antisense oligodeoxynucleotides, therefore, phosphorothioate oligodeoxynucleotides had a slower onset of action.

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EXAMPLE 8Concentration Dependence of Inhibition by Antisense bcl-2  
Phosphorothioate Oligodeoxynucleotides

5 The concentration dependence of inhibition by  
phosphorothioate and normal TI-AS oligodeoxynucleotides  
in cultures of 697 cells in serum-free medium was  
compared as follows.

10 697 cells were cultured in serum-free medium  
for either 3 days (normal oligodeoxynucleotides) or  
4 days (phosphorothioate oligodeoxynucleotides) prior to  
measuring cell densities and levels of DNA synthesis.  
Oligodeoxynucleotide additions to cultures included TI-AS  
phosphorothioate, TI-S phosphorothioate, TI-AS normal,  
and TI-S normal.

15 As shown in Figures 2 (a) and (b), TI-AS  
phosphorothioate oligodeoxynucleotides markedly inhibited  
the proliferation of 697 cells at 25-50  $\mu$ M. In contrast,  
normal TI-AS oligodeoxynucleotides required  
concentrations 5- to 10-fold higher (approximately 250  
20  $\mu$ M) to cause a comparable suppression of 697 cellular  
proliferation. Suppression by the antisense  
phosphorothioate oligodeoxynucleotide TI-AS was specific  
over this concentration range, since its complementary  
sense oligodeoxynucleotide (TI-S) produced little  
25 inhibition of 697 cell growth in replicate cultures (see  
Figures 2(a) and (b)).

EXAMPLE 9Influence of Serum Preparation on Inhibition by Antisense  
Phosphorothioate Oligodeoxynucleotides

30 To further define the effects of serum  
preparation on the inhibitory activity of  
phosphorothioate oligodeoxynucleotides, FBS that had been  
heated to 56°C for 30 minutes, 68°C for 1 hour, or not

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heated prior to addition to cultures was added to cultures of RS11846 lymphoma cells.

RS11846 cells were cultured in medium containing 1% (vol:vol) HL1-concentrate or 5% (vol:vol) FBS that had been heated at 56°C for 0.5 hour, 68°C for 1 hour, or that had not been heated. Cell counts were calculated as a percentage relative to control cultures treated with equivalent concentrations of TI-S phosphorothioate oligodeoxynucleotide, and represent the mean percentage (standard deviation was less than 10% for all values) for duplicate cultures counted on days 4 and 5.

The TI-AS phosphorothioate oligodeoxynucleotide completely inhibited the growth of RS11846 cells at 25  $\mu$ M, with an estimated half-maximal inhibitory concentration of approximately 11  $\mu$ M. In contrast, this phosphorothioate oligodeoxynucleotide was considerably less effective in cultures containing 5% (v:v) FBS. Furthermore, heating FBS prior to adding it to cultures did not significantly improve the ability of the TI-AS phosphorothioate oligodeoxynucleotide to suppress the growth of RS11846 lymphoma cells. At an oligodeoxynucleotide concentration of 50  $\mu$ M, inhibition of proliferation of RS11846 cells never exceeded 48% serum-containing cultures, regardless of the heating procedure used.

#### EXAMPLE 10

##### Influence of Dialysis of Serum on Inhibition by Normal and Phosphorothioate Antisense Oligodeoxynucleotides

To further characterize the nature of the interfering substances in serum, experiments were performed wherein 68°C-heated serum was extensively dialyzed (molecular weight cutoff = 3500) prior to being added to cultures of 697 leukemic cells. Experiments were conducted with 12.5  $\mu$ M TI-AS phosphorothioate

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oligodeoxynucleotide and 200  $\mu$ M of the normal oxygen-based TI-AS oligodeoxynucleotide.

697 cells were cultured in medium containing 1% (vol:vol) HL1-concentrate (A) or 5% (vol:vol) of three different lots of 68°C-treated FBS (B,C,D). Each serum preparation was contrasted before (ND) and after (D) extensive dialysis. TI-AS (+) and TI-S (-) oligodeoxynucleotides were added to replicate cultures at 200  $\mu$ M for normal oxygen-based oligodeoxynucleotides (OXY) and at 12.5  $\mu$ M for phosphorothioate oligodeoxynucleotides (PT). Relative levels of DNA synthesis (kcpm) were measured after 2 or 4 days of culture for normal and phosphorothioate oligodeoxynucleotides, respectively.

For the three different lots of FBS tested, two exhibited little change after dialysis in cultures containing either normal or phosphorothioate oligodeoxynucleotides. One lot of FBS, however, appeared to interfere less with the inhibitory activities of these antisense oligodeoxynucleotides after dialysis.

#### EXAMPLE 11

##### Experiments with Stably Transfected NIH 3T3 Cells

Though the antisense oligodeoxynucleotides described herein were designed to block *bcl-2* mRNA translation (TI-AS) and splicing (SD-AS), the molecular mechanisms of their actions are not yet known. To determine the effect of formation of oligodeoxynucleotide-RNA hybrids within cells upon inhibition of cellular growth, irrespective of the nucleotide sequence, cells transformed to express human *bcl-2* cDNA transcripts were cultured with normal oligodeoxynucleotides.

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200  $\mu$ M of normal TI-AS and TI-S oligodeoxynucleotides were added to cultures of typical NIH 3T3 cells and to cultures of these cells that had been stably transfected with expression constructs that produce high levels of human *bcl-2* cDNA transcripts for either the usual sense (3T3-alpha-S cells) or the antisense (3T3-alpha-AS cells) strand.

For RNA blot analyses, polyadenylated mRNA was purified from normal NIH 3T3 cells and from cells stably transfected with expression constructs that produce either sense (3T3-alpha-S) or antisense (3T3-alpha-AS) recombinant *bcl-2*-alpha mRNAs, according to the method of 13. Approximately 5  $\mu$ g of mRNA was subjected to RNA blot analysis, essentially as described in (16), using either <sup>32</sup>P-labeled hybridization probes derived from human or murine *bcl-2* sequences.

An autoradiogram resulting from a one-day exposure of a blot containing RNAs from normal 3T3 cells, 3T3-alpha-AS cells, and 3T3-alpha-S cells showed high relative levels of recombinant 2.4 and 1.4 kbp *bcl-2* transcripts produced from the *bcl-2* expression constructs that were transfected into 3T3-alpha-AS and 3T3-alpha-S cells.

A 10-day exposure of a blot containing RNA from normal 3T3 cells that were either proliferating or quiescent at the time of harvesting RNA showed low but detectable levels of normal 7.5 and 2.4 kbp murine *bcl-2* transcripts present in proliferating 3T3 cells.

TI-AS oligodeoxynucleotide specifically suppressed DNA synthesis and cellular replication in cultures of normal NIH 3T3 cells, consistent with findings by others that fibroblasts do contain *bcl-2* transcripts, albeit at low levels. The TI-AS

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oligodeoxynucleotide disclosed herein is complementary to the mouse *bcl-2* sequence in 18 of its 20 bases (17), accounting for its ability to suppress the growth of murine NIH 3T3 cells.

5                    NIH 3T3 cells, 3T3-alpha-AS cells, and  
3T3-alpha-S cells were cultured in medium containing 5%  
(vol:vol) 68°C-treated serum and either HBSS, 200  $\mu$ M TI-S  
normal oligodeoxynucleotide, or 200  $\mu$ M TI-AS normal  
oligodeoxynucleotide. Relative levels of DNA synthesis  
10 (kcpm) were measured in cultures after 3 days and reflect  
a 16 hour incubation with 0.5  $\mu$ ci/well of [<sup>3</sup>H]-thymidine.  
Cell densities, estimated by phase microscopy, were  
consistent with the measured DNA synthesis in cultures.  
The percentage of inhibition of DNA synthesis in cultures  
15 containing TI-AS oligodeoxynucleotides was calculated  
relative to control cultures containing HBSS.

As with normal NIH 3T3 cells, culturing  
3T3-alpha-S cells (producing human *bcl-2*-alpha sense  
transcripts) with TI-AS and TI-S oligodeoxynucleotides  
20 demonstrated specific suppression, since the sense  
oligodeoxynucleotide TI-S was not inhibitory. The level  
of inhibition of cellular proliferation by the antisense  
oligodeoxynucleotide, however, was not as great in  
3T3-alpha-S cells, as might be expected, since these  
25 cells contain more *bcl-2* mRNA.

Adding TI-S oligodeoxynucleotide to cultures of  
3T3-alpha-AS cells (produce antisense *bcl-2* transcripts)  
ruled out inhibition of cellular growth through a  
nonspecific mechanism involving oligodeoxynucleotide--RNA  
30 hybrid formation. The TI-S oligodeoxynucleotide caused  
little suppression of 3T3-alpha-AS cell proliferation,  
whereas the TI-AS oligodeoxynucleotide was markedly  
inhibitory in these cells. Similar data were obtained

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with TI-AS and TI-S phosphorothioate  
oligodeoxynucleotides.

EXAMPLE 12

Measurements of DNA Fragmentation as an Indicator of  
5 *bcl-2* Antisense Oligodeoxynucleotide-Mediated Programmed  
Cell Death in Human Lymphoma Cells

Oligonucleotides having the sequences shown in  
Table 2 were tested for the ability to induce programmed  
cell death (DNA fragmentation) in the human  
10 t(14:18)-containing human lymphoma cell line RS11846.  
The oligonucleotides were all phosphodiester, and were  
targeted against the translation initiation site or the  
5'-cap region of *bcl-2* pre-mRNAs. Control  
oligodeoxynucleotides included a *bcl-2* sense version  
15 (TI-S) of TI-AS (having SEQ ID NO: 7) and a scrambled  
version of TI-AS that has the same base composition, but  
with jumbled nucleotide order.

TABLE 2

	<u>SEQUENCE</u>	<u>SEQ ID NO:</u>
20	CGCGTGCGAC CCTCTTG	8
	TACCGCGTGC GACCCTC	9
	CCTTCCTACC GCGTGCG	11
	GACCCTTCCT ACCGCGT	12
	GGAGACCCTT CCTACCG	13
25	GCGGCGGCAG CGCGG	14
	CGGCGGGGCG ACGGA	15
	CGGGAGCGCG GCGGGC	16

RS11846 cells were adapted to grow in HL1 media  
with 1% FCS and their DNA metabolically labeled by  
30 addition of <sup>125</sup>I-deoxyuridine to cultures for three hours.  
Labeled cells were then washed thoroughly and cultured  
for two days in the presence of various oligonucleotides  
at 50 AM. Cells were then recovered from 200 µL cultures  
by centrifugation, and lysed in a hypotonic buffer

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containing 10 mM EDTA and 14 Triton X100. After centrifugation at 16,000 xg to pellet unfragmented genomic DNA, the supernatant fraction containing fragmented DNA was extracted with phenol/chloroform and ethanol precipitated. This DNA was then subjected to gel electrophoresis in 1.5% agarose gel and transferred to nylon membranes for autoradiography.

The results of two experiments are shown in Figures 3 and 4(a-b). The six *bcl-2* antisense oligonucleotides targeted in the vicinity of the ATG site of translation initiation in *bcl-2* mRNAs were tested. "C-Oligo-2" refers to an oligonucleotide with 4 purposeful mismatches. "U" indicates untreated control cells. Figures 4 (a-b) show the results for the oligonucleotides shown in Figure 3. "Sc20" refers to a 20 mer with the same base composition as TI-AS, but with scrambled sequence. Figure 4(b) shows the results for three oligonucleotides targeted against the 5'-cap of *bcl-2* mRNAs. The numbers refer to the distance of these oligomers from the ATG-translation initiation site.

The presence of a ladder of DNA fragments (unit size of approximately 200 bp) is indicative of programmed cell death. At 50  $\mu$ M, TI-AS caused little DNA fragmentation, whereas the oligonucleotides having SEQ ID NO: 9 and SEQ ID NO: 10, and one of the 5'-cap oligonucleotides (SEQ ID NO: 14) led to pronounced DNA fragmentation.

#### EXAMPLE 13

##### Concentration-Dependence of Inhibition by Antisense Phosphodiester Oligodeoxynucleotides in Serum-Free Cultures

697 pre-B cell leukemia cells were cultured in medium with either 1% (vol:vol) HL-1 concentrate



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(serum-free conditions [o] or 3% (vol:vol) 68°C-treated serum (FBS2) [\_\_\_], see Figure 5. Shown are cellular densities measured after 2 days in cultures containing various concentrations of phosphodiester TI-AS oligodeoxynucleotide. Data are shown as percentages relative to control cultures treated with a sense oligonucleotide, and reflect the mean  $\pm$  standard deviation for duplicate samples.

#### EXAMPLE 14

##### Immunofluorescence Analysis of bcl-2 Protein Levels in Oligodeoxynucleotide-Treated 697 Cells

For studies with oligodeoxynucleotides,  $0.25 \times 10^6$  (for phosphorothioate) or  $0.5 \times 10^5$  (for normal oligodeoxynucleotides), 697 cells were cultured in 1 ml of HL-1 serum-free medium in 24 well culture dishes (Linbro. Flow Lab, Inc.). After 2 days (for normal) or 4 days (for phosphorothioates), cells were recovered from cultures, washed once in [PBS, pH 7.4 (Gibco) - 0.1% bovine serum albumin - 0.1% sodium azide], and fixed for 5-10 minutes on ice in 1% paraformaldehyde/PBS solution. The cells were then washed once in PBS and incubated in 1 ml of absolute methanol at 20°C for 10 minutes. After washing once in PBS-A, cells were then resuspended in PBS containing 0.05% Triton-X100 for 3 minutes on ice, washed in PBS-A and preblocked for 30 minutes at 4°C in PBS with 10% (v/v) heat-inactivated goat serum.

For addition of the first antibody, preblocked cells were resuspended in 100  $\mu$ l of PBS-G (PBS-1% goat serum-0.1% sodium azide) prior to aliquoting 50  $\mu$ l into separate tubes that contained 1  $\mu$ l of either BCL2 antibody (Halder et al., Nature (London), 342:195-197 (1989)) or affinity-purified normal rabbit control IgG (Cappel 6012-0080) and incubated for 1 hour on ice. The BCL2 antibody used for these studies was prepared in rabbits using a synthetic peptide corresponding to amino

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acids (98-114) of the BCL2 protein and was affinity--  
purified by protein-A-Sepharose chromatography and used  
at approximately 1 mg/ml. Cells were then washed in  
PBS-A and incubated in 0.5-1.0 ml PBS-A for 15-20 minutes  
on ice to allow diffusion of nonspecific cell-associated  
antibody prior to resuspending cells in 100  $\mu$ l of PBS-G  
containing 5  $\mu$ g of biotinylated scat anti-rabbit IgG  
(BA1000; Vector Labs) for 30 minutes. After washing once  
and incubating for 15 minutes in PBS-A, cells were  
finally resuspended in 100  $\mu$ l of PBS-A containing 2  $\mu$ g of  
FITC-conjugated avidin (Vector Labs A2011) for 20 minutes  
and washed three times in PBS-A prior to analysis with an  
Ortho cytofluorograph 50-H connected to an Ortho 2150  
data-handling system. The specificity of method for  
detecting BCL2 protein was confirmed by  
immunofluorescence microscopy (showing cytosolic stain  
peptide competition, and studies of cell lines that  
expressed various levels of BCL2 mRNA and proteins  
through gene transfer manipulations.

For measurements of surface HLA-DR antigen  
expression, an indirect immunofluorescence assay method  
was used (Reed et al., *J. Immunol.* 134:1631-1639 (1985))  
involving incubation of viable cells with a murine  
anti-HLA-DR monoclonal antibody (IgG2a) (Becton-Dickinson  
7360) or a negative control antibody, R3-367 (IgG2a),  
followed by FITC-conjugated scat anti-mouse IgG (Cappel  
1711-0081). Cells were fixed in 1% paraformaldehyde/PBS  
prior to FACS analysis.

697 cells were cultured for 2 days (PO) or  
4 days (PS) with various oligonucleotides. In  
Figures 6(a-b), the black columns show the results with a  
sense oligonucleotide, and the hatched columns with an  
antisense oligonucleotide TI-AS. Cells were labeled with  
anti-bcl-2 antiserum and analyzed by FACS. Data are

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expressed as percentages relative to the mean fluorescence obtained with untreated 697 cells.

Figures 7(a-d) show typical FACS results obtained for 697 cells before and after treatment with 100  $\mu$ M PO *bcl-2* antisense oligonucleotides. A: untreated 697 cells labeled with either anti-*bcl-2* antiserum (hatched area) or normal rabbit serum control (white area); B: untreated 697 cells labeled with either anti-HLA-DR antibody (hatched area) or a negative control antibody (white area); C: 697 cells cultured for 2 days with either normal *bcl-2* TI-AS (white area) or TI-AS (hatched area) oligodeoxynucleotides and labeled with anti-*bcl-2* antibody; D: 697 cells cultured with TI-AS and TI-S oligodeoxynucleotides (as in C), but labeled with anti-HLA-DR antibody.

As shown in Figures 6 (a) and (b), PO and PS *bcl-2* antisense oligonucleotides produced specific concentration-dependent reductions in the levels of *bcl-2* proteins, without altering the levels of expression of HLA-DR (Figures 7(a-d)) and other control antigens. At 150  $\mu$ M, for example, PO antisense oligodeoxynucleotide caused an approximately 75-95% reduction in *bcl-2* fluorescence, whereas the control sense oligodeoxynucleotide diminished *bcl-2* protein levels by only 10-20% (Figure 6(a)). Similarly, cultured 697 cells for 4 days with the PS antisense oligodeoxynucleotide at 25  $\mu$ M resulted in approximately 70% reduction in *bcl-2* fluorescence. In comparison, the sense PS oligodeoxynucleotide TI-AS inhibited *bcl-2* protein levels by only approximately 15%, as measured by this assay (Figure 6(b)).

#### SIGNIFICANCE

In phosphorothioate oligodeoxynucleotides, one of the non-bridging oxygen atoms in each internucleotide

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phosphate linkage is replaced by a sulfur atom. This modification renders phosphorothioate oligodeoxynucleotides extremely resistant to cleavage by nucleases, Stein et al., *Nucl. Acids Res.*, 16:3209-3221 (1988). Despite the substitution of a sulfur atom for an oxygen, phosphorothioate oligodeoxynucleotides retain good solubility in aqueous solutions; hybridize well, though with some decrease in the melting temperature of RNA-oligodeoxynucleotides duplexes; and are synthesized conveniently by the widely employed method of automated oligodeoxynucleotides synthesis with phosphoroamidites.

Antisense *bcl-2* phosphorothioate oligodeoxynucleotides have been found to be more potent inhibitors of leukemic cell growth than their normal oxygen-based counterparts. When tested under serum-free conditions, these oligodeoxynucleotides reduced cellular proliferation by half at concentrations of approximately 15-23  $\mu\text{M}$ , whereas the normal oligodeoxynucleotide achieved 50% inhibition at 125-250  $\mu\text{M}$ . This finding may be explained by the reduced sensitivity of phosphorothioate oligodeoxynucleotides to cellular nucleases, or may be attributable to other mechanisms. For example, mRNAs hybridized with phosphorothioate oligodeoxynucleotides may experience enhanced degradation through a mechanism involving an RNase H-like activity.

Despite their increased inhibitory activity, phosphorothioate antisense oligodeoxynucleotides retained sequence-specificity. At the concentrations tested (less than 25  $\mu\text{M}$ ), sense versions of these oligodeoxynucleotides had little effect on leukemic cell growth. Both normal and phosphorothioate antisense oligodeoxynucleotides appeared to initially suppress the proliferation of leukemic cells through non-cytotoxic mechanisms. During the first few days of culture, cellular replication was inhibited without a concomitant

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rise in cell death. Later in these cultures (days 4-5 for normal oligodeoxynucleotides, days 6-8 for phosphorothioates), however, cellular viabilities declined.

5           Comparing the kinetics of inhibition by normal and phosphorothioate oligodeoxynucleotides revealed that the latter compounds have a slower onset of action. Maximal inhibition of leukemic cell proliferation by  
10       normal antisense oligodeoxynucleotides occurred two days after initiation of cultures, whereas phosphorothioate oligodeoxynucleotides required 4 to 7 days to achieve maximal inhibition.

          The usefulness of anticode oligomers in inhibiting human lymphoma/leukemia cells and other types  
15       of cancer cells that express the *bcl-2* gene has been shown by the examples herein. Anti-sense oligodeoxynucleotides complementary to at least an effective portion of the mRNA of the human *bcl-2* gene has  
20       been found to inhibit growth of RS11846 human follicular lymphoma cells t (14;18) chromosomal translocation and high *bcl-2* expression), 697 human pre B cell leukemia cells (high *bcl-2* expression), JURKAT human acute lymphocytic leukemia cells (medium *bcl-2* expression),  
25       normal human lymphocytes (medium *bcl-2* expression) and murine fibroblasts (low *bcl-2* expression). Although *bcl-2* antisense reagents can suppress the growth of many types of cells, the t(14:18) lymphoma and leukemia cells seem to be the sensitive, allowing for specific inhibition of malignant cells.

30           As demonstrated in the following Examples, a variety of DNA analogs can be employed in the instant invention. For example, phosphorothioates, methylphosphonates, and mixed oligomers containing combinations of phosphodiester and phosphorothioate or

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methylphosphonate nucleosides. It should be understood that RNA analogs can also be employed in the invention.

#### EXAMPLE 15

##### Methylphosphonate (MP)/Phosphodiester (PO) bcl-2

##### Antisense Oligomers Induce Death of DoHH2 Lymphoma Cells

The purpose of this study was to determine the efficacy of various analogs of the anticode oligomers for inhibiting lymphoma cell survival.

DoHH2 is a human lymphoma cell line containing a t(14:18)-translocation that activates the bcl-2 gene. DoHH2 cells were cultured for 3 days without oligomers or in the presence of various concentrations of antisense (As) and scrambled (Sc) methylphosphonate (MP)/Phosphodiester (PO) oligomers for 3 days. Cell viability was assessed by trypan blue dye exclusions, and the data expressed as a percentage relative to DoHH2 cells cultured without oligomers. The MP/PO oligomers was an 18-mer targeted against the first 6 codons of the bcl-2 open reading frame in which 5 internal linkages were phosphodiester and the flanking nucleosides were methylphosphonates.

The results indicate that these anticode oligomer analogs are potent and specific inhibitors of lymphoma cell survival.

25

#### EXAMPLE 16

##### Methylphosphonate (MP)/Phosphodiester (PO) Chimeric

##### Oligomers Inhibit Growth of MCF-7

##### Human Breast Cancer Cells

The purpose of this study was to determine the efficacy of the claimed anticode oligomer analogs to inhibit the survival of solid tumor cells which highly express bcl-2.

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MCF-7 is a human breast adenocarcinoma cell line that contains relatively high levels of bcl-2 protein. The cells were cultured at 4,000 cells per well in 96-well microtiter plates in the presence or absence of MP/PO oligomers. Relative cell numbers per well were then estimated by MTT assay, based on a standard curve prepared using freshly plated, untreated MCF-7 cells. The antisense (As) and scrambled (Sc) MP/PO oligomers were the same as those described in Example 15. Data represent the mean +/- standard deviation for determinations.

The results demonstrate sequence specific inhibition of growth of solid tumor cells by the the claimed anticode oligomer analogs.

#### EXAMPLE 17

##### Optimization of Anticodons bcl-2 Oligomer Sequences

The purpose of this study was to determine optimum target sites or sequence portions on mRNA for inhibiting cell survival by contacting the cells with various claimed anticodons molecules whose sequences were computer generated.

DoHH2 lymphoma cells were treated with various concentrations of oligomers targeted to different sites on the bcl-2 mRNA. The ATG oligomer targets the translation initiation site, and is complementary to the first 6 codons of the open reading frame. The Dscore 23 and Dscore 72 oligomers target sites in the 5' untranslated region of the mRNA. Sc oligomers represent negative controls having the same length and base composition but in scrambled order. All oligomers were prepared as phosphodiester (PO)/phosphorothioate (PS) chimeras, where only the last (3') two internucleoside linkages were phosphorothioates. Oligomers were added directly to cultures and relative numbers of viable cells

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were estimated by MTT assay 3 days later. Data represent mean +/- standard deviation.

The results indicate that the Dscore 23 oligomer, targeted to the 5' untranslated region, has, compared to the other anticode oligomers tested in this Example, superior activity for inhibiting cell survival.

#### EXAMPLE 18

##### Reveral of Chemoresistance of Tumor Cells by Antisense-Mediated Reduction of bcl-2 Gene Expression

The following work was undertaken to determine if anticode oligomers directed against the expression of the bcl-2 gene would reverse chemoresistance, that is to say, increase the sensitivity to cancer chemotherapeutic agents in cancer tumor cells expressing the bcl-2 gene.

High levels of bcl-2 protein appeared to increase the relative resistance of lymphoid cells to killing induced by a wide variety of cancer chemotherapeutic agents including, but not limited to, Ara-C, MTX, vincristine, taxol, cisplatin, adriamycin, etoposide, mitozantron, 2-chlorodeoxyadenosine, dexamethasone (DEX), and alkylating agents. (Miyashita, T. and Reed, J.C., Cancer Res. 52:5407, October 1, 1992). While these drugs have diverse biochemical mechanisms of action, it is believed that all have in common the ability to ultimately trigger cancer cell death by activating endogenous cellular pathways leading to apoptosis (Eastman, A. Cancer Cells 2:275 (1990)). It is understood that the claimed anticode molecules and analogs thereof as used herein are effective for their intended purposes of enhancing sensitivity to cancer chemotherapeutic drugs including, but not limited to, antimetabolites, alkylating agents, plant alkaloids, and antibiotics.



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Antimetabolites include, but are not limited to, methotrexate, 5-fluoruracil, 6-mercaptopurine, cytosine arabinoside, hydroxyurea, 20chlorodeoxy adenosine.

5 Alkylating agents include, but are not limited to, cyclophosphamide, melphalan, busulfan, cisplatin, paraplantin, chlorambucil, and nitrogen mustards.

Plant alkaloids include, but are not limited to, vincristine, vinblastine, VP-16.

10 Antibiotics include, but are not limited to, doxorubicin (adriamycin), daunorubicin, mitomycin c, bleomycin.

Other cancer chemotherapeutic agents include DTIC (decarbazine), mAMSA, hexamethyl melamine, 15 mitroxantrone, taxol, etoposide, dexamethasone.

In the present work, both nuclease resistance phosphorothioates (PS) and phosphodiester in which only the 3'-most internucleoside bond was a thioate linkage (PO/PS) were employed. The PO/PS oligomers are 20 resistance to 3' exonucleases (the principal nuclease activity of serum) and generally form more stable heteroduplexes with target RNAs.

Cationic lipids were used to improve the uptake and subsequent release of oligomers into effective 25 intracellular compartments, and are exemplary pharmaceutical carriers for the claimed anticode oligomers.

The methods for preparing and purifying the antisense (AS) and scrambled (SC) 18' mer oligonucleotides 30 used for the present work are described above in General

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Methods and in Kitada et al. (Antisense R & D, 3:157 (1993)). Phosphodiester oligonucleotides were synthesized in a 10-15 micromole scale using phosphoroamidate chemistry with oxidation by iodine, and then purified using a C<sub>18</sub>-reverse phase column. In most cases, oligomers were additionally ethanol-precipitated five times to eliminate any nonspecific cytotoxic activity, and then dried and resuspended in sterile HL-1 medium (Ventrex Labs, Inc; Burlingame, CA) at 1-10 mM. The pH of this solution was adjusted using 1-10 M NaOH until the phenol red indicator dye in the media returned to its original color.

The principal oligomers used were 18-mers, having either the sequence:

I. TCTCCCAGCGTGCGCCAT (SEQ ID NO. 17), which is antisense to the first six codons of the human bcl-2 open reading frame (SEQ ID NO. 19); or

II. TGCACTCACGCTCGGCCT (SEQ ID NO. 18), which is a scrambled version used as a control.

Standard transfection methods were used to produce tumor cells expressing either the bcl-2 gene or an antisense oligodeoxynucleotide which bound to bcl-2 mRNA. It is understood that the vector could also encode an antisense oligodeoxynucleotide which binds to bcl-2 pre-mRNA. The particular nucleotide sequence encoding the antisense oligonucleotides of the invention is not critical, except that the sequences are preferably chosen such that they express antisense oligodeoxynucleotides sufficient to reduce bcl-2 gene expression in tumor cells and increase the sensitivity of the tumor cells to cancer chemotherapeutic agents or sufficient to kill tumor cells when they are treated with cancer chemotherapeutic agents. It is only necessary that the antisense oligodeoxynucleotide encoded in vector is expressed under

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conditions sufficient to reduce bcl-2 gene expression in tumor cells. The methods used for preparing vectors, and, in particular, expression plasmids, for transferring genes into mammalian cells relies on routine techniques in the field of molecular biology. A basic text disclosing the general methods of preparing expression plasmids used in this invention is Molecular Cloning, A Laboratory Manual, 2nd Edition, eds. Sambrook et al., Cold Spring Harbor Laboratory Press, (1989), particularly Chapter 16 on Expression of Cloned Genes in Cultured Mammalian Cells. Examples 15C-D below set forth particular methods for preparing the expression plasmids used in the present invention. The particular vector used to transfer the antisense oligonucleotides of the present invention is not critical, and such vectors may include vectors derived from lambda and related phages or from filamentous phages. It is only necessary that the transferred nucleotide sequence encoding the antisense oligonucleotides of the present invention be expressed in the transfected tumor cell under conditions sufficient to reduce bcl-2 gene expression in the tumor cell. The present invention includes expression of the antisense oligonucleotide either from an extrachromosomal position (e.g. from an expression plasmid) or from a position integrated into the host genome itself, as mediated by other vectors, such as recombinant retroviral vectors (Reed et al. bcl-2 mediated tumorigenicity in a T-cell lymphoid cell line: synergy with C-MYC and inhibition by bcl-2 antisense. PNAS USA 87:3660 (1990)).

A. Treatment of Lymphoma Cells With 18-mer Synthetic bcl-2 Antisense Oligodeoxynucleotides.

Lymphoma cell line SU-DHL-4, obtained from a use of diffuse, histiocytic, non-Hodgins lymphoma (Epstein et al. Two new monoclonal antibodies (LN-1, LN-2) reactive in B5 formalin-fixed, paraffin-embedded

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tissues with follicular center and mantle zone human B lymphocytes and derived tumors. J. Immunol. 133:1028 (1984)) and containing a t(14;18) translocation was treated with 18-mer synthetic bcl-2-AS  
5 oligodeoxynucleotides targeted for binding with the first six codons of the bcl-2 mRNA. As a control, SU-DHL-4 cells were treated with various control oligomers, including 18-mers having the same nucleoside composition as the AS oligomer, but in which the bases were in  
10 scrambled order (SC).

Aliquots of 1.5 ml of HL-1 serum-free medium (Ventrex Labs, Inc.) supplemented with 1 mM L-glutamine, 50 Units/ml penicillin, and 100 ug/ml streptomycin and either 5 ug of purified oligonucleotides or 30 ug of  
15 Lipofectin<sup>R</sup> [1:1 w/w mixture of N-(1-2,3-dioleyloxy)propyl)-n,n,n-trimethylammonium chloride (DOTMA) and dioleoylphosphatidylethanolamine (DOPE)] were combined and added to  $0.75 \times 10^6$  SU-DHL-4 cells in 3 mls of HL-1 medium. Cells were then either  
20 cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air in 24 well plates (2 mls/well) for immunoblot and RT-PCR assays, or in 96-well flat-bottom microtiter plates (0.1 ml/well) for MTT assays. For cells in  
25 microtiter cultures, typically 0.1 ml of additional HL-1 media with or without various chemotherapeutic drugs was added after 1 day, and the cells were cultured for an additional 2 days before performing MTT assays.

Cells were washed once in PBS, lysed in a buffer containing 1% Triton X100, and samples normalized  
30 for protein content (25 ug) prior to size-fractionation of proteins by SDS-PAGE (12% gels) and transfer to nitrocellulose filters for immunoblot assays as described in Reed et al. Cancer Res. 51:6529 (1991). Preliminary experiments determined that aliquots of lysates  
35 containing 25 ug of total protein produced results in the

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linear range of the assay. Blots were first incubated with 0.1% (v.v) of a rabbit antiserum directed against a synthetic peptide corresponding to amino-acids (aa) 41-54 of the human Bcl-2 protein, as shown in SEQ ID NO. 21 (id) followed by 2.8 ug/ml biotinylated goat anti-rabbit IgG (Vector Labs, Inc.). Bands corresponding to p26-Bcl-2 were then visualized by color development using a Horseradish Peroxidase (HRP)-avidin-biotin complex reagent (Vector Labs, Inc) and 3,3'-diaminobenzidine (DAB). Stained blots were then incubated with a second anti-Bcl-2 antibody directed against aa 61-76 of the Bcl-2 protein (SEQ ID NO. 21) followed by 0.25 uCi/ml <sup>125</sup>I-protein A. Bcl-2 bands were excised from the blots and subjected to gamma-counting.

Despite the mitochondrial location of Bcl-2 protein, no difference in the rate of MTT dye reduction by mitochondrial enzymes was noted in cells that were identical except for their levels of p26-Bcl-2. These comparisons were made using pairs of exponentially growing lymphoid cell lines that differed only in that one line had been stably infected with a recombinant bcl-2 retrovirus and the other with the parental retroviral vector lacking a bcl-2 cDNA insert (Miyashita et al. Cancer Res. 52:5407 (1992); Blood 81:151 (1993)).

Anticodons specific reductions in the relative levels of bcl-2 mRNA were detected within 1 day by a semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay. See Figure 8A.

SU-DHL-4 cells were cultured with 0.83 ug/ml of oligomers complexed with 5 ug of cationic lipids (Lipofectin; BRL/Gibco, Inc.) per ml of serum-free media Y(13,19). In Figure 8A, total RNA was isolated from cells after 1 day and relative levels of bcl-2 and glyceraldehyde 3'-phosphate dehydrogenase (GAPDH) mRNAs

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were assessed by RT-PCR assay as described in Kitada et al. Antisense R & D 3:157 (1993)).

In Figure 8(b), SU-DHL-4 cells were cultured with pairs of either PS (squares) or PO/PS (circles) As- and Sc-oligomers for 3 days. Relative levels of Bcl-2 protein were then measured using a quantitative immunoblot assay, as described above, and the data expressed as a percentage relative to cells treated with control Sc-oligomers. The inset shows immunoblot results for p26-Bcl-2 and a p75 cross-reactive (CR) band in a case where As-PO/PS oligomer produced a 41% relative decrease in Bcl-2 protein levels. In Figure 8(c),  $10^{-6}$ M Ara-C, MTX, or DEX was added 1 day after addition of PS (squares) or PO/PS (circles) oligomers to cultures of SU-DHL-4 cells, and MTT assays were performed on day 3. Data are presented as a % control relative to cells cultured with drugs in the absence of any oligomers, and represent the results of 9 of 10 consecutive experiments [in one experiment, the MTT assay failed]. Similar results were obtained when dye exclusion assays were used to assess cell survival rather than MTT assay [not shown].

Mean values for the data are indicated by horizontal lines. Statistical analysis of the data was by paired t-test (As versus Sc). Concentrations of As- and Sc-oligomers ( $\approx 150$  nM) were adjusted to maximize As effects while maintaining sequence specificity.

Variations in the amounts of starting RNA were controlled for by RT-PCR analysis using primers specific for GAPDH mRNA.

The long half-life of the bcl-2 protein (approximately 14 hours) may account for the AS-mediated reductions in bcl-2 proteins not being as dramatic as for

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reductions in bcl-2 mRNA, taking longer to achieve (about 3 days), and appearing more variable.

Figure 8(c) shows the composite results for 10 experiments where relative levels of bcl-2 protein were compared in SU-DHL-4 cells treated with AS or SC oligomers. AS-mediated reductions in bcl-2 protein levels ranged from as much as 66% to as little as 10%, with an average relative reduction of about 30%, compared to SU-DHL-4 cells that were treated in the identical manner with control oligomers. Levels of a variety of control mitochondrial proteins such as F<sub>1</sub>-beta-ATPase and cytochrome C, which like bcl-2 are encoded by nuclear genes, were not adversely affected by AS-oligomers (not shown), indicating that the AS-mediated reductions in bcl-2 protein levels were specific. The insert in Figure 8(b), for example, shows a comparison of p26-Bcl-2 with a 78-kDa protein that cross reacts with one of the rabbit antisera employed for immunoblot assays, demonstrating a decrease in the levels of p26-bcl-2 but not p78 in the AS-treated cells relative to cells that received control SC-oligomers.

B. Effect of Treatment of SU-DHL-4 Cells with bcl-2 AS Oligomers on Cell Sensitivity to Cancer Chemotherapeutic Agents

This study was performed to determine whether treatment of SU-DHL-4 cells with bcl-2 AS-oligomers could increase their relative sensitivity to killing by the cancer chemotherapeutic agents Ara-C, MTX, and DEX, which are anticancer drugs.

Previous control studies demonstrated that bcl-2 AS oligomers had little or no effect on SU-DHL-4 cell growth and survival at least during the first three days of culture (Kitada et al. Antisense R & D 3:157 (1993)). AS-mediated reductions in bcl-2 protein levels in these

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lymphoma cells as well as in other cells do not typically accelerate the rate of cell death in cultures unless the cells are deprived of serum growth factors (Reed et al. Proc. Natl. Acad. Sci. USA 87:3660 (1990)).

5           In the present work, preliminary studies demonstrated that more than 90% of SU-DHL-4 cells survived treatment for 4 days with high dose ( $10^{-4}$ ) Ara-C, MTX or DEX, presumably because of their high levels of bcl-2 protein (Not shown). At these concentrations,  
10           however, all drugs induced essentially complete inhibition of SU-DHL-4 cell proliferation, consistent with bcl-2 converts drugs from cytotoxic to cytostatic. Comparisons of AS and SC oligomers demonstrated that bcl-2 AS treatment markedly enhanced the sensitivity of these  
15           lymphoma cells to MTX and Ara-C, and to a lesser extent to DEX (Figure 8(c)).

          Despite some variability in results, on average, the addition of bcl-2 AS oligomers to cultures of SU-DHL-4 cells treated with MTX or Ara-C resulted in  
20           79-84% greater inhibition (reduction in viable cell numbers) than use of either drug alone ( $P < 0.002$  for AS versus SC) in the absence of introducing the bcl-2 AS oligomers of the invention. Statistically significant results were obtained for DEX-treated SU-DHL-4 cells  
25           ( $P=0.01$ ). The 20-30% reduction in viable cell numbers observed for control oligomer-treated cells could reflect a degree of sequence non-specificity, but was probably related to the use of cationic lipids to facilitate oligomer delivery into cells.



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C. Effect of Transfecting Cells With Expression Plasmids Encoding Human bcl-2 Protein on Sensitivity to Chemotherapeutic Agents.

To further confirm the sequence specificity of bcl-2 AS oligomers for enhancing sensitivity to chemotherapeutic anticancer drugs, a study was conducted using an Interleukin-3 (IL-3)-dependent murine hemopoietic cell line 32D.C13 that had been stably transfected with expression plasmid encoding either the human bcl-2 protein or a viral homolog of bcl-2, BHRF-1, which has only 22% homology with bcl-2. 32D.C13 cells were obtained from Dr. Giovanni Rovera of the Wistar Institute, Philadelphia, PA.

Treatment of 32D cells with oligomer/cationic lipid complexes was as described above except that 50 Units/ml of murine recombinant IL-3 (rIL-3) was included in the HL-1 media, the initial cell density was  $10^5$  per ml, and replication-defective adenovirus dl312 (MOI=200) was added 30 minutes after exposure of cells to oligomers to facilitate exit of DNA from endosomes [Yoshimura K, et al. J Biol Chem. 268, 2300, (1993)].

32D cells that had been stable transfected with expression plasmids encoding either human p26-Bcl-2 or EBV p19-BHRF-1 (Takayama, S. et al. submitted) were cultured in medium ( $10^5$ /ml) containing IL-3 and PO/PS oligomers for 3 days to achieve reductions in human Bcl-2 protein levels. The cells were then retreated with oligomers alone (C) or in combination with various concentrations of MTX and the relative number of viable cells assessed by MTT assay 2 days later. Data represent mean  $\pm$  standard deviation for triplicate determinations and are expressed as a % relative to cells that received no MTX. Statistical analysis of data for  $10^{-6}$  to  $10^{-4}$  M MTX was by a 2-way Analysis of Variables method (Finney, D.J. In Statistical Methods in Biological Assays, p. 72,

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1978 (3rd edition, Charles Griffin & Co., London).  
Comparable results were obtained with dye exclusion  
assays [not shown].

5 RNAs derived from the human bcl-2 construct in  
32D-BCL-2 cells were a target for bcl-2 AS oligomers,  
whereas RNAs from the BHRF-1 expression plasmid are not.  
Thus the chemosensitivity to cytotoxic drugs of 32D.C13  
cells expressing BHRF-1 should have been unaffected by  
the AS treatment.

10 Preliminary experiments demonstrated that upon  
withdrawal of IL-3 from 32D.C13 cells, levels of  
endogenous mouse bcl-2 protein declined and the cells  
underwent apoptosis. bcl-2 and BHRF-1 comparably  
supported the survival of 32D.C13 cells in the absence of  
15 IL-3, and the proliferative rates of 32D.C13 cells  
containing high levels of these proteins were similar in  
the presence of IL-3, thus excluding these variables as  
explanations for any differences in chemosensitivity.

20 Figures 9(a) and (b) compares the sensitivity  
of 32D-BCL-2 and 32D-BHRF-1 cells to various  
concentrations of MTX. Treatment with bcl-2 AS-oligomers  
resulted in sequence-specific increases in the  
sensitivity of 32D-BCL-2 cells to inhibition by MTX at  
concentrations of  $10^{-6}$  to  $10^{-4}$  M ( $P \leq 0.001$  for AS versus  
25 SC). In contrast, treatment with bcl-2 AS oligomers  
produced no significant difference in the sensitivity of  
32D-BHRF-1 cells to MTX, relative to control SC-oligomers  
(Figures 9(a) and (b)). These data indicate that the  
effects of bcl-2 AS oligomers on chemosensitivity to  
30 cytotoxic agents drugs are sequence specific. Furthermore,  
several other control oligomer, including bcl-2 sense,  
other scrambled sequences with the same nucleoside  
composition as AS, and oligomers with totally unrelated

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s quences all had comparatively little effect on the chemosensitivity of the cells (Not shown).

The findings above demonstrated that bcl-2 AS oligomers produced sequence specific reductions in bcl-2 mRNA and bcl-2 protein levels and that these events were associated with increased sensitivity to chemotherapeutic agents such as anticancer drugs. The portion of tumor cells killed by the chemotherapeutic agents was greater than the portion killed by the same amount of chemotherapeutic agents in the absence of introducing the bcl-2 AS oligomers of the invention.

D. Effect of Transfecting Cells With Expression Plasmids Encoding Human bcl-2 Protein on Sensitivity of Lymphoma Cells to Chemotherapeutic Agents.

A different strategy was employed to determine if AS-mediated reductions in bcl-2 gene expression could be achieved with an inducible bcl-2 AS expression plasmid that used a heavy metal responsive human metallothionein-IIA promoter in another translocation t(14;18)-containing lymphoma line, RS11846. RS11846 was obtained from Dr. Carlo Croce (Wistar Institute, Philadelphia, PA (Tsujimoto and Croce, Proc. Natl. Acad. Sci. USA 83:5214 (1986)).

To prepare the expression plamid, a 0.91 kbp bcl-2 cDNA (ibid)) was subcloned in either antisense (AS) or sense (S) orientatino into a HindIII site downstream of a human metallothionein-IIA promoter in the plasmid pMEP-4 (Invitrogen, Inc.), which contains a hygromycin phosphotransferase gene and the EBNA-1 gene and origin of DNA replication from Epstein Varr Virus for high copy episomal maintenance.

RS11846 cells ( $5 \times 10^6$ ) in Dulbecco's phosphate buffered saline containing 30 ug of plasmid DNA were

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electroporated (1500 uF, 270 V/cm) using a Cellject Electroporation System from EquiBio, Inc. Cells were returned to their usual culture media (RPMI-L 1640 supplemented with 10% fetal bovine serum, 1 mM L-glutamine, 50 Units/ml penicillin, and 100 ug/ml streptomycin) at  $2 \times 10^5$  cells per ml and cultured for 2 days before seeding cells at  $2 \times 10^5$  per ml in media containing 200 ug/ml hygromycin. After 3 weeks of culture, the resulting bulk cell lines were passaged in successively higher concentrations of hygromycin in 200 ug/ml increments until the concentration reached 1 mg/ml (about 4 weeks).

Hygromycin-resistant RS11846 cell were cultured in RPMI/10% serum media containing 0.5 uM  $\text{CdCl}_2$  and 3 days later immunoblot assays were performed using 25 ug protein/lane essentially as described in Tanaka S, et al. *J. Biol. Chem.* 268, 10920 (1993) and in Reed et al. *Cancer Res.* 51:6529 (1991)).

As summarized in Figures 10(a), (b)(1), (b)(2), and (b)(3), control ("C") and *bcl-2*-As ("As") plasmids were introduced into RS11846 cells and expression was induced with either 0.5 uM  $\text{CdCl}_2$  or 50 uM  $\text{ZnCl}_2$  for various times. As an additional control, RS11846 cells containing inducible plasmids with the *bcl-2* cDNA in sense ("S") orientation were also analyzed. RS11846 cells were induced for 3 days and relative levels of Bcl-2 and  $\text{F}_1\text{-}\beta\text{-ATPase}$  proteins were assessed by immunoblot assay of Tanaka et al. *J. Biol. Chem.* 268:10920 (1993). In Figure 10(a), RS11846 cells were cultured at  $10^5$  cells/ml in medium containing 0.5 uM  $\text{CdCl}_2$  and 1 day later  $10^{-7}$  M Ara-C or an equivalent volume of diluent control was added. Relative numbers of viable cells were estimated from MTT assays at various times and the mean  $\pm$  S.D. calculated for triplicate samples. In Figures 10(b)(1-3), RS11846 cells were cultured as in Figure

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10(a), except that various concentrations of Ara-C, MTX, or DEX were added. Data represent mean +/- S.D. for triplicate samples. Statistical calculations are by 2-way Analysis of Variables. DEX served as a negative control here since RS11846 cells have lost glucocorticoid receptors.

Preliminary experiments demonstrated that RS11846 cells tolerated the addition of up to 0.5 microm CdCl<sub>2</sub> or to microm ZnCl<sub>2</sub> to cultures for one week, experiencing a slight decrease in growth rate but essentially no decline in percentage cell viability (Not shown).

In the absence of heavy metal induction, the relative levels of bcl-2 protein in RS11846 cells containing the control or bcl-2 AS plasmid were comparable, as determined by immunoblot assays (Not shown). When 0.5 uM CdCl<sub>2</sub> or uM ZnCl<sub>2</sub> was added, reductions in bcl-2 protein became evident in the AS-expressing cells at 2 days and maximal inhibition of 30-40% was obtained at three to four days, relative to control RS11846 cells.

Figure 10(a) shows an example of immunoblot data derived from RS11846 cells after three days of exposure to 0.5 mM CdCl<sub>2</sub>, demonstrating reduced levels of bcl-2 protein in the AS-plasmid containing cells compared to RS11846 cells that harbored the control plasmid. The relative levels of a control mitochondrial protein F<sub>1</sub>-beta-ATPase were comparable in all cell lines, consistent with sequence-specific alterations in bcl-2 protein levels.

When RS11846 cells containing either the control or bcl-2-As plasmids were cultured for various times in 0.5uM CdCl<sub>2</sub> or 50 uM ZnCl<sub>2</sub>, no significant

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difference in the growth rates of these two cell lines was observed (Figure 8(b)). Thus, As-mediated reductions in Bcl-2 protein levels by themselves did not impair RS11846 cell proliferation or survival.

5                   Inclusion of low-dose Ara-C ( $10^{-7}$ M) in the cultures of control RS11846 cells resulted in only a slight decline in the net numbers of viable cells, presumably because of the high levels of Bcl-2 protein found in these t(14;18)-containing lymphoma cells. In  
10 contrast, addition of  $10^{-7}$ M Ara-C to cultures of bcl-2-AS expressing RS11846 cells was markedly inhibitory (Figure 8(b)). Ara-C, however, had no effect on bcl-2 AS-expressing RS11846 cells in the absence of heavy metal induction of the MT promoter, when directly compared with  
15 RS11846 cells containing the control plasmid under the same conditions [not shown]. Figure 8(c) shows that the enhanced sensitivity to Ara-C observed for bcl-2-AS-expressing RS11846 cells occurred over a wide range of drug concentrations ( $P < 0.001$ ). Heavy-metal  
20 induction of the bcl-2-AS expression plasmid also significantly increased the relative sensitivity of RS11846 lymphoma cells to MTX ( $P < 0.001$ ), but not to DEX. Glucocorticoid receptor binding assays demonstrated that RS11846 cells have lost receptors for these steroid  
25 hormones [not shown], thus providing a specificity control showing that AS-mediated reductions in bcl-2 protein levels are by themselves insufficient to impair the growth or survival of these lymphoma cells.

30                   Using a plurality of anticode approaches, the present invention demonstrated that average reductions of 30-40% in the relative levels of bcl-2 protein markedly enhanced the sensitivity of lymphoma cells, in particular, t(14;18)-containing lymphoma cell lines to chemotherapeutic agents such as conventional anticancer  
35 drugs. These examples demonstrated that introducing the

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claimed anticode oligomers into tumor cells achieves a reduction of *bcl-2* expression and increases the chemosensitivity of neoplastic cells to chemotherapeutic agents or anticancer drugs.

5                   Accordingly, the present invention achieved a method of killing tumor cells by introducing to tumor cells anticode oligomers which reduce *bcl-2* gene expression or impair *Bcl-2* protein function before  
10                   contacting the cells with chemotherapeutic agents including anticancer drugs. The conventional anticancer drugs reduced the numbers of viable malignant cells, and the portion of tumor cells killed was greater than the portion which would have been killed by the same amount  
15                   of drug in the absence of introducing the anticode oligomers into the cells.

                  Having thus disclosed exemplary embodiments of the present invention, it should be noted by those skilled in the art that this disclosure is exemplary only and that various other alternatives, adaptations, and  
20                   modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein, but is only limited by the following claims.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Reed, John
- (ii) TITLE OF INVENTION: Regulation of bcl-2 Gene Expression
- (iii) NUMBER OF SEQUENCES: 23
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Gray, Cary, Ames & Frye
  - (B) STREET: 401 B Street, Suite 1700
  - (C) CITY: San Diego
  - (D) STATE: CA
  - (E) COUNTRY: USA
  - (F) ZIP: 92101-4297
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Diskette 3.5 inch, 1.44 Mb storage
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: US
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Brotman, Harris F.
  - (B) REGISTRATION NUMBER: 35461
  - (C) REFERENCE/DOCKET NUMBER: P0041US0
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (619) 699-3630
  - (B) TELEFAX: (619) 236-1048

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iv) ANTI-SENSE: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCCTTCCTAC CGCGTGCGAC

20



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## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTTTTCCTCT GGGAAGGATG GCGCACGCTG GGAGA

35

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCTCCGACCC ATCCACGTAG

20

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACGGGGTACG GAGGCTGGGT AGGTGCATCT GGT

33

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## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTTGACGTCC TACGGAAACA

20

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCCCAACTG CAGGATGCCT TTGTGGAAGT GTACGG

36

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGGAAGGATG GCGCAGCTG

20

## (2) INFORMATION FOR SEQ ID NO:8:

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- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 17 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGCGTGCGAC CCTCTTG

17

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 17 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TACCGCGTGC GACCCTC

17

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 17 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCCTACCGCG TGC GACC

17

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 17 base pairs
  - (B) TYPE: nucleic acid

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- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCTTCCTACC GCGTGCG

17

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 17 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GACCCTTCCT ACCGCGT

17

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 17 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGAGACCCTT CCTACCG

17

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 15 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCGGCGGCAG CGCGG

15

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGGCGGGGCG ACGGA

15

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGGGAGCGCG GCGGGC

16

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TCTCCCAGCG TGCGCCAT

18

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TGCACTCACG CTCGGCCT

18

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## (2) INFORMATION FOR SEQ ID NO:19:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5086 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCGCCCGCCC CTCCGCGCCG CCTGCCCGCC CGCCCGCCGC GCTCCCGCCC GCCGCTCTCC	60
GTGGCCCCGC CGCGCTGCCG CCGCCGCCGC TGCCAGCGAA GGTGCCGGGG CTCCGGGCCC	120
TCCCTGCCGG CGGCCGTCAG CGCTCGGAGC GAACTGCGCG ACGGGAGGTC CGGGAGGCGA	180
CCGTAGTCGC GCCGCCGCGC AGGACCAGGA GGAGGAGAAA GGGTGCGCAG CCCGGAGGCG	240
GGGTGCGCCG GTGGGGTGCA GCGGAAGAGG GGGTCCAGGG GGGAGAACTT CGTAGCAGTC	300
ATCCTTTTTA GGAAAAGAGG GAAAAAATAA AACCTCCCC CACCACCTCC TTCTCCCCAC	360
CCCTCGCCGC ACCACACACA GCGCGGGCTT CTAGCGCTCG GCACCGGCGG GCCAGGCGCG	420
TCCTGCCTTC ATTTATCCAG CAGCTTTTCG GAAAATGCAT TTGCTGTTTC GAGTTTAATC	480
AGAAGACGAT TCCTGCCTCC GTCCCCGGCT CCTTCATCGT CCCATCTCCC CTGTCTCTCT	540
CCTGGGGAGG CGTGAAGCGG TCCCGTGGAT AGAGATTCAT GCCTGTGTCC GCGCGTGTGT	600
GCGCGCGTAT AAATTGCCGA GAAGGGGAAA ACATCACAGG ACTTCTGCGA ATACCGGACT	660
GAAAATTGTA ATTCATCTGC CGCCGCCGCT GCCAAAAAAA AACTCGAGCT CTTGAGATCT	720
CCGGTTGGGA TTCCTGCGGA TTGACATTTT TGTGAAGCAG AAGTCTGGGA ATCGATCTGG	780
AAATCCTCCT AATTTTACT CCCTCTCCCC CCGACTCCTG ATTCATTGGG AAGTTTCAA	840
TCAGCTATAA CTGGAGAGTG CTGAAGATTG ATGGGATCGT TGCCTTATGC ATTTGTTTTG	900
GTTTTACAAA AAGGAACTT GACAGAGGAT CATGCTGTAC TTAAAAATA CAAGTAAGTC	960
TCGCACAGGA AATTGGTTTA ATGTAACTTT CAATGGAAAC CTTTGAGATT TTTTACTTAA	1020
AGTGCATTCG AGTAAATTTA ATTTCCAGGC AGCTTAATAC ATTGTTTTTA GCCGTGTTAC	1080
TTGTAGTGTG TATGCCCTGC TTTCACTCAG TGTGTACAGG GAAACGCACC TGATTTTTTA	1140
CTTATTAGTT TGTTTTTTCT TTAACCTTTC AGCATCACAG AGGAAGTAGA CTGATATTAA	1200
CAATACTTAC TAATAATAAC GTGCCTCATG AAATAAAGAT CCGAAAGGAA TTGGAATAAA	1260
AATTCCTGCT GTCTCATGCC AAGAGGGAAA CACCAGAATC AAGTGTTCCG CGTGATTGAA	1320

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GACACCCCCT	CGTCCAAGAA	TGCAAAGCAC	ATCCAATAAA	ATAGCTGGAT	TATAACTCCT	1380
CTTCTTTCTC	TGGGGGCCGT	GGGGTGGGAG	CTGGGGCGAG	AGGTGCCGTT	GGCCCCCGTT	1440
GCTTTTCCTC	TGGGAAGGAT	GGCGCACGCT	GGGAGAACGG	GGTACGACAA	CCGGGAGATA	1500
GTGATGAAGT	ACATCCATTA	TAAGCTGTCT	CAGAGGGGCT	ACGAGTGGGA	TGCGGGAGAT	1560
GTGGGCGCCG	CGCCCCCGGG	GGCCGCCCCC	GCACCGGGCA	TCTTCTCCTC	CCAGCCCCGG	1620
CACACGCCCC	ATCCAGCCGC	ATCCCGCGAC	CCGGTCGCCA	GGACCTCGCC	GCTGCAGACC	1680
CCGGCTGCCC	CCGGCGCCGC	CGCGGGGCCT	GCGCTCAGCC	CGGTGCCACC	TGTGGTCCAC	1740
CTGGCCCTCC	GCCAAGCCGG	CGACGACTTC	TCCCGCCGCT	ACCGCGGCGA	CTTCGCCGAG	1800
ATGTCCAGCC	AGCTGCACCT	GACGCCCTTC	ACCGCGCGGG	GACGCTTTGC	CACGGTGGTG	1860
GAGGAGCTCT	TCAGGGACGG	GGTGAAGTGG	GGGAGGATTG	TGGCCTTCTT	TGAGTTCGGT	1920
GGGGTCATGT	GTGTGGAGAG	CGTCAACCGG	GAGATGTCGC	CCCTGGTGGA	CAACATCGCC	1980
CTGTGGATGA	CTGAGTACCT	GAACCGGCAC	CTGCACACCT	GGATCCAGGA	TAACGGAGGC	2040
TGGGATGCCT	TTGTGGAAGT	GTACGGCCCC	AGCATGCGGC	CTCTGTTTGA	TTTCTCCTGG	2100
CTGTCTCTGA	AGACTCTGCT	CAGTTTGGCC	CTGGTGGGAG	CTTGATCAC	CCTGGGTGCC	2160
TATCTGAGCC	ACAAGTGAAG	TCAACATGCC	TGCCCCAAAC	AAATATGCAA	AAGGTTCACT	2220
AAAGCAGTAG	AAATAATATG	CATTGTCACT	GATGTACCAT	GAAACAAAGC	TGCAGGCTGT	2280
TTAAGAAAAA	ATAACACACA	TATAAACATC	ACACACACAG	ACAGACACAC	ACACACACAA	2340
CAATTAACAG	TCTTCAGGCA	AAACGTCGAA	TCAGCTATTT	ACTGCCAAAG	GGAAATATCA	2400
TTTATTTTTT	ACATTATTAA	GAAAAAAGAT	TTATTTATTT	AAGACAGTCC	CATCAAAACT	2460
CCGTCTTTGG	AAATCCGACC	ACTAATTGCC	AAACACCGCT	TCGTGTGGCT	CCACCTGGAT	2520
GTTCTGTGCC	TGTAAACATA	GATTGCTTTT	CCATGTTGTT	GGCCGGATCA	CCATCTGAAG	2580
AGCAGACGGA	TGGAAAAAGG	ACCTGATCAT	TGGGGAAGCT	GGCTTTCTGG	CTGCTGGAGG	2640
CTGGGGAGAA	GGTGTTTATT	CACCTGCATT	TCTTTGCCCT	GGGGGCGTGA	TATTAACAGA	2700
GGGAGGGTTC	CCGTGGGGGG	AAGTCCATGC	CTCCCTGGCC	TGAAGAAGAG	ACTCTTTGCA	2760
TATGACTCAC	ATGATGCATA	CCTGGTGGGA	GGAAAAGAGT	TGGGAACTTC	AGATGGACCT	2820
AGTACCCACT	GAGATTTCCA	CGCCGAAGGA	CAGCGATGGG	AAAAATGCCC	TTAAATCATA	2880
GGAAAGTATT	TTTTTAAGCT	ACCAATTGTG	CCGAGAAAAG	CATTTTAGCA	ATTTATACAA	2940
TATCATCCAG	TACCTTAAAC	CCTGATTGTG	TATATTCATA	TATTTTGGAT	ACGCACCCCC	3000

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CAACTCCCAA	TACTGGCTCT	GTCTGAGTAA	GAAACAGAAT	CCTCTGGAAC	TTGAGGAAGT	3060
GAACATTTTCG	GTGACTTCCG	ATCAGGAAGG	CTAGAGTTAC	CCAGAGCATC	AGGCCGCCAC	3120
AAGTGCCTGC	TTTTAGGAGA	CCGAAGTCCG	CAGAACCTAC	CTGTGTCCCA	GCTTGGAGGC	3180
CTGGTCCTGG	AACTGAGCCG	GGCCCTCACT	GGCCTCCTCC	AGGGATGATC	AACAGGGTAG	3240
TGTGGTCTCC	GAATGTCTGG	AAGCTGATGG	ATGGAGCTCA	GAATTCCACT	GTCAAGAAAG	3300
AGCAGTAGAG	GGGTGTGGCT	GGGCCTGTCA	CCCTGGGGCC	CTCCAGGTAG	GCCCGTTTTTC	3360
ACGTGGAGCA	TAGGAGCCAC	GACCCTTCTT	AAGACATGTA	TCACTGTAGA	GGGAAGGAAC	3420
AGAGGCCCTG	GGCCTTCCTA	TCAGAAGGAC	ATGGTGAAGG	CTGGGAACGT	GAGGAGAGGC	3480
AATGGCCACG	GCCCATTTTTG	GCTGTAGCAC	ATCGCACGTT	GGCTGTGTGG	CCTTGGCCAC	3540
CTGTGAGTTT	AAAGCAAGGC	TTTAAATGAC	TTTGGAGAGG	GTCACAAATC	CTAAAAGAAG	3600
CATTGAAGTG	AGGTGTCATG	GATTAATTGA	CCCCTGTCTA	TGGAATTACA	TGTAAAACAT	3660
TATCTTGTC	CTGTAGTTTG	GTTTTATTTG	AAAACCTGAC	AAAAAAAAAAG	TTCCAGGTGT	3720
GGAATATGGG	GGTTATCTGT	ACATCCTGGG	GCATTAAAAA	AAAATCAATG	GTGGGGAACT	3780
ATAAAGAAGT	AACAAAAGAA	GTGACATCTT	CAGCAAATAA	ACTAGGAAAT	TTTTTTTTTCT	3840
TCCAGTTTAG	AATCAGCCTT	GAAACATTGA	TGGAATAACT	CTGTGGCATT	ATTGCATTAT	3900
ATACCATTTA	TCTGTATTAA	CTTTGGAATG	TACTCTGTTC	AATGTTTAAT	GCTGTGGTTG	3960
ATATTTTCGAA	AGCTGCTTTA	AAAAAATACA	TGCATCTCAG	CGTTTTTTTTG	TTTTTAATTG	4020
TATTTAGTTA	TGGCCTATAC	ACTATTTGTG	AGCAAAGGTG	ATCGTTTTCT	GTTTGAGATT	4080
TTTATCTCTT	GATTCTTCAA	AAGCATTCTG	AGAAGGTGAG	ATAAGCCCTG	AGTCTCAGCT	4140
ACCTAAGAAA	AACCTGGATG	TCACTGGCCA	CTGAGGAGCT	TTGTTTCAAC	CAAGTCATGT	4200
GCATTTCCAC	GTCAACAGAA	TTGTTTATTG	TGACAGTTAT	ATCTGTTGTC	CCTTTGACCT	4260
TGTTTCTTGA	AGGTTTCCTC	GTCCCTGGGC	AATTCCGCAT	TTAATTCATG	GTATTCAGGA	4320
TTACATGCAT	GTTTGGTTAA	ACCCATGAGA	TTCATTGAGT	TAAAAATCCA	GATGGCGAAT	4380
GACCAGCAGA	TTCAAATCTA	TGGTGGTTTG	ACCTTTAGAG	AGTTGCTTTA	CGTGGCCTGT	4440
TTCAACACAG	ACCCACCCAG	AGCCCTCCTG	CCCTCCTTCC	GCGGGGGCTT	TCTCATGGCT	4500
GTCCTTCAGG	GTCTTCCTGA	AATGCAGTGG	TCGTTACGCT	CCACCAAGAA	AGCAGGAAAC	4560
CTGTGGTATG	AAGCCAGACC	TCCCCGGCGG	GCCTCAGGGA	ACAGAATGAT	CAGACCTTTG	4620
AATGATTCTA	ATTTTTAAGC	AAAATATTAT	TTTATGAAAG	GTTTACATTG	TCAAAGTGAT	4680

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GAATATGGAA	TATCCAATCC	TGTGCTGCTA	TCCTGCCAAA	ATCATTTTAA	TGGAGTCAGT	4740
TTGCAGTATG	CTCCACGTGG	TAAGATCCTC	CAAGCTGCTT	TAGAAGTAAC	AATGAAGAAC	4800
GTGGACGTTT	TTAATATAAA	GCCTGTTTTG	TCTTTTGTTG	TTGTTCAAAC	GGGATTCAAC	4860
GAGTATTTGA	AAAATGTATA	TATATTAAGA	GGTCACGGGG	GCTAATTGCT	AGCTGGCTGC	4920
CTTTTGCTGT	GGGGTTTTGT	TACCTGGTTT	TAATAACAGT	AAATGTGCCC	AGCCTCTTGG	4980
CCCCAGAACT	GTACAGTATT	GTGGCTGCAC	TTGCTCTAAG	AGTAGTTGAT	GTTGCATTTT	5040
CCTTATTGTT	AAAAACATGT	TAGAAGCAAT	GAATGTATAT	AAAAGC		5086

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## (2) INFORMATION FOR SEQ ID NO:20:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 717 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..717

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATG GCG CAC GCT GGG AGA ACG GGG TAC GAC AAC CGG GAG ATA GTG ATG	48
Met Ala His Ala Gly Arg Thr Gly Tyr Asp Asn Arg Glu Ile Val Met	
1 5 10 15	
AAG TAC ATC CAT TAT AAG CTG TCG CAG AGG GGC TAC GAG TGG GAT GCG	96
Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala	
20 25 30	
GGA GAT GTG GGC GCC GCG CCC CCG GGG GCC GCC CCC GCA CCG GGC ATC	144
Gly Asp Val Gly Ala Ala Pro Pro Gly Ala Ala Pro Ala Pro Gly Ile	
35 40 45	
TTC TCC TCC CAG CCC GGG CAC ACG CCC CAT CCA GCC GCA TCC CGC GAC	192
Phe Ser Ser Gln Pro Gly His Thr Pro His Pro Ala Ala Ser Arg Asp	
50 55 60	
CCG GTC GCC AGG ACC TCG CCG CTG CAG ACC CCG GCT GCC CCC GGC GCC	240
Pro Val Ala Arg Thr Ser Pro Leu Gln Thr Pro Ala Ala Pro Gly Ala	
65 70 75 80	
GCC GCG GGG CCT GCG CTC AGC CCG GTG CCA CCT GTG GTC CAC CTG GCC	288
Ala Ala Gly Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Ala	
85 90 95	
CTC CGC CAA GCC GGC GAC GAC TTC TCC CGC CGC TAC CGC GGC GAC TTC	336
Leu Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Gly Asp Phe	
100 105 110	
GCC GAG ATG TCC AGC CAG CTG CAC CTG ACG CCC TTC ACC GCG CGG GGA	384
Ala Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly	
115 120 125	
CGC TTT GCC ACG GTG GTG GAG GAG CTC TTC AGG GAC GGG GTG AAC TGG	432
Arg Phe Ala Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp	
130 135 140	

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GGG Gly 145	AGG Arg	ATT Ile	GTG Val	GCC Ala	TTC Phe 150	TTT Phe	GAG Glu	TTC Phe	GGT Gly	GGG Gly 155	GTC Val	ATG Met	TGT Cys	GTG Val	GAG Glu 160	480
AGC Ser	GTC Val	AAC Asn	CGG Arg	GAG Glu 165	ATG Met	TCG Ser	CCC Pro	CTG Leu	GTG Val 170	GAC Asp	AAC Asn	ATC Ile	GCC Ala	CTG Leu 175	TGG Trp	528
ATG Met	ACT Thr	GAG Glu	TAC Tyr 180	CTG Leu	AAC Asn	CGG Arg	CAC His	CTG Leu 185	CAC His	ACC Thr	TGG Trp	ATC Ile	CAG Gln 190	GAT Asp	AAC Asn	576
GGA Gly	GGC Gly	TGG Trp 195	GAT Asp	GCC Ala	TTT Phe	GTG Val	GAA Glu 200	CTG Leu	TAC Tyr	GGC Gly	CCC Pro	AGC Ser 205	ATG Met	CGG Arg	CCT Pro	624
CTG Leu 210	TTT Phe	GAT Asp	TTC Phe	TCC Ser	TGG Trp	CTG Leu 215	TCT Ser	CTG Leu	AAG Lys	ACT Thr	CTG Leu 220	CTC Leu	AGT Ser	TTG Leu	GCC Ala	672
CTG Leu 225	GTG Val	GGA Gly	GCT Ala	TGC Cys	ATC Ile 230	ACC Thr	CTG Leu	GGT Gly	GCC Ala	TAT Tyr 235	CTG Leu	AGC Ser	CAC His	AAG Lys		717

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## (2) INFORMATION FOR SEQ ID NO:21:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 239 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```

Met Ala His Ala Gly Arg Thr Gly Tyr Asp Asn Arg Glu Ile Val Met
 1           5           10           15
Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala
          20           25           30
Gly Asp Val Gly Ala Ala Pro Pro Gly Ala Ala Pro Ala Pro Gly Ile
          35           40           45
Phe Ser Ser Gln Pro Gly His Thr Pro His Pro Ala Ala Ser Arg Asp
          50           55           60
Pro Val Ala Arg Thr Ser Pro Leu Gln Thr Pro Ala Ala Pro Gly Ala
 65           70           75           80
Ala Ala Gly Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Ala
          85           90           95
Leu Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Gly Asp Phe
          100          105          110
Ala Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly
          115          120          125
Arg Phe Ala Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp
          130          135          140
Gly Arg Ile Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu
          145          150          155          160
Ser Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp
          165          170          175
Met Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn
          180          185          190
Gly Gly Trp Asp Ala Phe Val Glu Leu Tyr Gly Pro Ser Met Arg Pro
          195          200          205
Leu Phe Asp Phe Ser Trp Leu Ser Leu Lys Thr Leu Leu Ser Leu Ala
          210          215          220

```

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Leu Val Gly Ala Cys Ile Thr Leu Gly Ala Tyr Leu Ser His Lys  
 225 230 235

## (2) INFORMATION FOR SEQ ID NO:22:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 615 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..615

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATG GCG CAC GCT GGG AGA ACG GGG TAC GAC AAC CGG GAG ATA GTG ATG	48
Met Ala His Ala Gly Arg Thr Gly Tyr Asp Asn Arg Glu Ile Val Met	
1 5 10 15	
AAG TAC ATC CAT TAT AAG CTG TCG CAG AGG GGC TAC GAG TGG GAT GCG	96
Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala	
20 25 30	
GGA GAT GTG GGC GCC GCG CCC CCG GGG GCC GCC CCC GCA CCG GGC ATC	144
Gly Asp Val Gly Ala Ala Pro Pro Gly Ala Ala Pro Ala Pro Gly Ile	
35 40 45	
TTC TCC TCC CAG CCC GGG CAC ACG CCC CAT CCA GCC GCA TCC CGC GAC	192
Phe Ser Ser Gln Pro Gly His Thr Pro His Pro Ala Ala Ser Arg Asp	
50 55 60	
CCG GTC GCC AGG ACC TCG CCG CTG CAG ACC CCG GCT GCC CCC GGC GCC	240
Pro Val Ala Arg Thr Ser Pro Leu Gln Thr Pro Ala Ala Pro Gly Ala	
65 70 75 80	
GCC GCG GGG CCT GCG CTC AGC CCG GTG CCA CCT GTG GTC CAC CTG GCC	288
Ala Ala Gly Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Ala	
85 90 95	

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CTC	CGC	CAA	GCC	GGC	GAC	GAC	TTC	TCC	CGC	CGC	TAC	CGC	GGC	GAC	TTC	336
Leu	Arg	Gln	Ala	Gly	Asp	Asp	Phe	Ser	Arg	Arg	Tyr	Arg	Gly	Asp	Phe	
			100					105					110			
GCC	GAG	ATG	TCC	AGC	CAG	CTG	CAC	CTG	ACG	CCC	TTC	ACC	GCG	CGG	GGA	384
Ala	Glu	Met	Ser	Ser	Gln	Leu	His	Leu	Thr	Pro	Phe	Thr	Ala	Arg	Gly	
		115					120					125				
CGC	TTT	GCC	ACG	GTG	GTG	GAG	GAG	CTC	TTC	AGG	GAC	GGG	GTG	AAC	TGG	432
Arg	Phe	Ala	Thr	Val	Val	Glu	Glu	Leu	Phe	Arg	Asp	Gly	Val	Asn	Trp	
		130					135				140					
GGG	AGG	ATT	GTG	GCC	TTC	TTT	GAG	TTC	GGT	GGG	GTC	ATG	TGT	GTG	GAG	480
Gly	Arg	Ile	Val	Ala	Phe	Phe	Glu	Phe	Gly	Gly	Val	Met	Cys	Val	Glu	
					150					155					160	
AGC	GTC	AAC	CGG	GAG	ATG	TCG	CCC	CTG	GTG	GAC	AAC	ATC	GCC	CTG	TGG	528
Ser	Val	Asn	Arg	Glu	Met	Ser	Pro	Leu	Val	Asp	Asn	Ile	Ala	Leu	Trp	
				165					170					175		
ATG	ACT	GAG	TAC	CTG	AAC	CGG	CAC	CTG	CAC	ACC	TGG	ATC	CAG	GAT	AAC	576
Met	Thr	Glu	Tyr	Leu	Asn	Arg	His	Leu	His	Thr	Trp	Ile	Gln	Asp	Asn	
			180					185					190			
GGA	GGC	TGG	GTA	GGT	GCA	TCT	GGT	GAT	GTG	AGT	CTG	GGC				615
Gly	Gly	Trp	Val	Gly	Ala	Ser	Gly	Asp	Val	Ser	Leu	Gly				
		195					200					205				

## (2) INFORMATION FOR SEQ ID NO:23:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 205 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met	Ala	His	Ala	Gly	Arg	Thr	Gly	Tyr	Asp	Asn	Arg	Glu	Ile	Val	Met
1				5					10					15	
Lys	Tyr	Ile	His	Tyr	Lys	Leu	Ser	Gln	Arg	Gly	Tyr	Glu	Trp	Asp	Ala
			20					25					30		
Gly	Asp	Val	Gly	Ala	Ala	Pro	Pro	Gly	Ala	Ala	Pro	Ala	Pro	Gly	Ile
		35					40					45			
Phe	Ser	Ser	Gln	Pro	Gly	His	Thr	Pro	His	Pro	Ala	Ala	Ser	Arg	Asp
	50				55						60				
Pro	Val	Ala	Arg	Thr	Ser	Pro	Leu	Gln	Thr	Pro	Ala	Ala	Pro	Gly	Ala
65					70				75					80	

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Ala	Ala	Gly	Pro	Ala	Leu	Ser	Pro	Val	Pro	Pro	Val	Val	His	Leu	Ala
				85					90					95	
Leu	Arg	Gln	Ala	Gly	Asp	Asp	Phe	Ser	Arg	Arg	Tyr	Arg	Gly	Asp	Phe
			100					105					110		
Ala	Glu	Met	Ser	Ser	Gln	Leu	His	Leu	Thr	Pro	Phe	Thr	Ala	Arg	Gly
		115					120					125			
Arg	Phe	Ala	Thr	Val	Val	Glu	Glu	Leu	Phe	Arg	Asp	Gly	Val	Asn	Trp
	130					135					140				
Gly	Arg	Ile	Val	Ala	Phe	Phe	Glu	Phe	Gly	Gly	Val	Met	Cys	Val	Glu
145					150					155					160
Ser	Val	Asn	Arg	Glu	Met	Ser	Pro	Leu	Val	Asp	Asn	Ile	Ala	Leu	Trp
				165					170					175	
Met	Thr	Glu	Tyr	Leu	Asn	Arg	His	Leu	His	Thr	Trp	Ile	Gln	Asp	Asn
			180					185					190		
Gly	Gly	Trp	Val	Gly	Ala	Ser	Gly	Asp	Val	Ser	Leu	Gly			
		195					200					205			

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CLAIMS:

1. An anticodon oligomer which binds to a site in mRNA transcribed from the human bcl-2 gene, said site being a strategic site selected from the splice donor site, the splice acceptor site and the or a portion of the 5'-untranslated region of said mRNA, which anticodon oligomer, when brought into contact with cancer cells expressing the human bcl-2 gene, has the property of reducing the expression of at least one bcl-2 gene product and thereby inducing programmed cell death of said cancer cells.
2. An anticodon oligomer as claimed in claim 1 wherein said anticodon oligomer is an antisense oligonucleotide or analogue thereof.
3. An anticodon oligomer as claimed in claim 1 or claim 2 wherein said anticodon oligomer is substantially complementary to said strategic site.
4. An anticodon oligomer as claimed in any one of the preceding claims wherein said strategic site is in the 5'-untranslated region of said mRNA expressed from the human bcl-2 gene and wherein said anticodon oligomer binds exclusively to the or a portion of the 5'-untranslated region.
5. An anticodon oligomer as claimed in any one of claims 2 to 4 wherein said anticodon oligomer is a phosphodiester/methylphosphonate chimera or a phosphodiester/phosphorothioate chimera or a methylphosphonate/phosphorothioate chimera.
6. An anticodon oligomer as claimed in any one of claims 2 to 4 wherein said anticodon oligomer or chirally enriched form thereof is comprised of one or more groups selected from methylphosphonates,

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phosphorothioates, diesters, phosphorodithioates, or 2' substituted derivatives.

5           7.    An anticodon oligomer as claimed in any one of the preceding claims which is two to two hundred nucleotide bases long.

8.    An anticodon oligomer as claimed in any one of the preceding claims which is ten to forty nucleotide bases long.

10       9.    An anticodon oligomer as claimed in any one of the preceding claims wherein the cells expressing the human bcl-2 gene are human tumor cells.

10.   An anticodon oligomer as claimed in claim 8 wherein the strategic site is a sequence portion in pre-mRNA expressed from said bcl-2 gene.

15       11.   An anticodon oligomer as claimed in claim 10 wherein said anticodon oligomer is substantially complementary to and binds to a strategic site in said pre-mRNA.

20       12.   An anticodon oligomer as claimed in claim 10 or claim 11 wherein said strategic site in said pre-mRNA is selected from the translation initiation site, the splice donor site, the splice acceptor site, codons 1 to 6 of the bcl-2 open reading frame and the or a portion of the 5'-untranslated region.

25       13.   An anticodon oligomer as claimed in any one of claims 9 to 12 which is two to two hundred nucleotide bases long.

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14. An anticode oligomer as claimed in any one of claims 9-13 which is ten to forty nucleotide bases long.

5 15. An anticode oligomer as claimed in any one of claims 9 to 13 wherein the cells expressing the human bcl-2 gene are human tumor cells.

10 16. An anticode oligomer useful for inhibiting the growth of cells expressing the human bcl-2 gene which anticode oligomer binds with a strategic site of RNA expressed from the human bcl-2 gene, which anticode oligomer is a phosphodiester/methylphosphonate chimera or a phosphodiester/phosphorothioate chimera or a methylphosphonate/phosphorothioate chimera of an antisense oligonucleotide which when brought into contact  
15 with cancer cells expressing the human bcl-2 gene, has the property of reducing the expression of at least one bcl-2 gene product and thereby inducing programmed cell death of said cancer cells.

20 17. An anticode oligomer useful for inhibiting the growth of cells expressing the human bcl-2 gene comprising an anticode oligomer which binds with a strategic site of RNA expressed from the human bcl-2 gene, which anticode oligomer or chirally enriched form thereof is comprised of one or more groups selected from  
25 methylphosphonates, phosphorothioates, diesters, phosphorodithioates, or 2' substituted derivatives, which anticode oligomer when brought into contact with cancer cells expressing the human bcl-2 gene, has the property of reducing the expression of at least one bcl-2 gene  
30 product and thereby inducing programmed cell death of said cancer cells.

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18. A vector for transfecting human cancer cells comprising a nucleotide sequence that encodes an anticodon oligomer as claimed in any one of claims 1 to 4.

5 19. A vector for transfecting human cancer cells comprising a nucleotide sequence that encodes an anticodon oligomer which reduces expression from the human bcl-2 gene in said cells, said vector being derived from a phage.

10 20. A composition useful for inhibiting the growth of cells expressing the human bcl-2 gene which comprises an anticodon oligomer as claimed in any one of claims 1 to 17 together with a pharmaceutically acceptable carrier.

15 21. A composition useful for inhibiting the growth of cells expressing the human bcl-2 gene which comprises a vector as claimed in claim 18 or claim 19 and a pharmaceutically acceptable carrier.

20 22. A composition as claimed in claim 20 wherein said pharmaceutically acceptable carrier is a cationic lipid.

23. A composition as claimed in claim 20 wherein said anticodon oligomer is encapsulated in liposomes.

25 24. Use of an anticodon oligomer as claimed in any one of claims 1 to 17 in the manufacture of a medicament for treating human cancer.

25. Use of a vector as claimed in claim 18 or claim 19 in the manufacture of a medicament for treating human cancer.

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26. Use of an anticode oligomer which binds with a sequence portion of mRNA expressed from the human bcl-2 gene in the manufacture of a medicament for treating solid tumors in humans.

5           27. Use of an anticode oligomer which binds with a sequence portion of mRNA expressed from the human bcl-2 gene in the manufacture of a medicament for treating breast cancer.

10           28. Materials for simultaneous or sequential use for killing human cancer cells expressing the bcl-2 gene comprising:

an anticode oligomer which binds to a sequence portion of mRNA expressed from a human bcl-2 gene and;

15           a chemotherapeutic agent toxic to said human cancer cells.

20           29. Materials as claimed in claim 28 wherein said chemotherapeutic agent is selected from antimetabolites, alkylating agents, plant alkaloids and antibiotics.

30. Materials as claimed in claim 29 wherein said chemotherapeutic agent further comprises a radionuclide.

25           31. Materials as claimed in claim 28 or claim 29 wherein said anticode oligomer is as claimed in any one of claims 1 to 17.

32. A method of inducing programmed cell death of cancer cells which express the human bcl-2 gene comprising the steps of:

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- a. providing an anticode oligomer as claimed in any one of claims 1 to 17; and
- b. contacting said cells with said anticode oligomer under conditions sufficient to induce programmed cell death in said cells.

33. A method of killing human cancer cells which express the human bcl-2 gene comprising the steps of:

- a. providing an anticode oligomer which binds with a sequence portion of mRNA expressed from the human bcl-2 gene;
- b. contacting said cells with said anticode oligomer under conditions to inhibit growth of said cells; and
- c. contacting said cells with an amount of at least one secondary therapeutic agent toxic to said cells sufficient to kill at least a portion thereof.

34. A method as claimed in claim 33 wherein said anticode oligomer is as claimed in any one of claims 1 to 17.

35. A method as claimed in claim 33 wherein said secondary agent is a chemotherapeutic agent.

36. A method as claimed in any one of claims 33-35 wherein said chemotherapeutic agent is selected from antimetabolites, alkylating agents, plant alkaloids and antibiotics.

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37. A method as claimed in claims 33 to 34 wherein said secondary therapeutic agent is a radiotherapeutic agent.

5 38. A method as claimed in claims 33, 34, or 37 in which said step of contacting said cells with a radiotherapeutic agent comprises contacting said cells with a beam of radiation, wherein said radiation comprises either particle radiation or non-particle radiation generated from a source which is non-contiguous  
10 with said cells.

39. A method as claimed in claims 33, 34, or 37 in which said step of contacting said cells with a radiotherapeutic agent comprises contacting said cells with a radionuclide.

15 40. A composition useful for killing human cancer cells expressing the bcl-2 gene which comprises an anticode oligomer as claimed in any one of claims 1-17 and a chemotherapeutic agent toxic to said human cancer cells.

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FIG. 1(a)

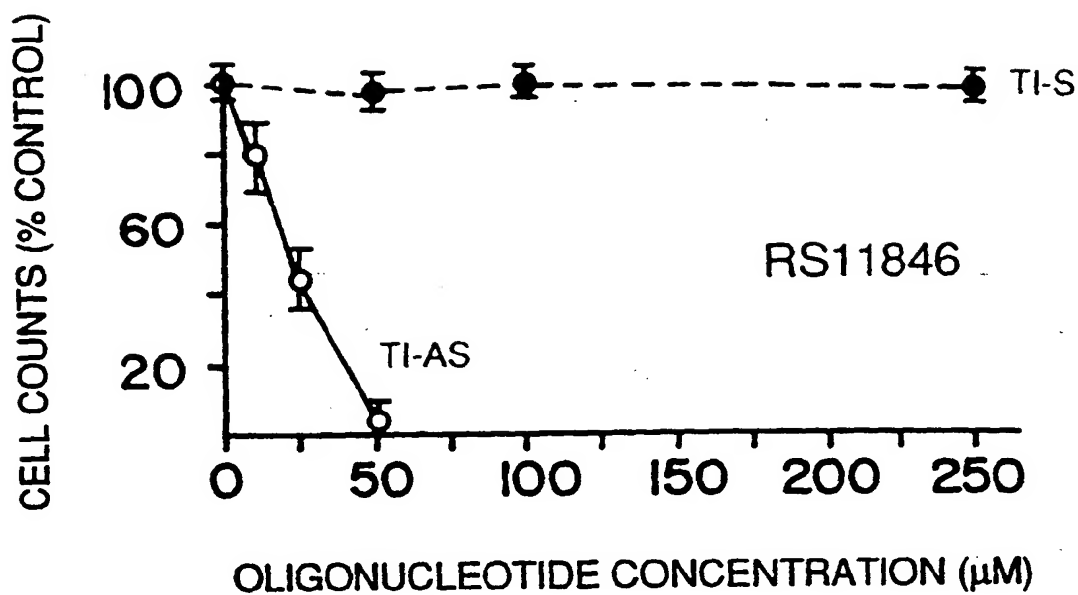
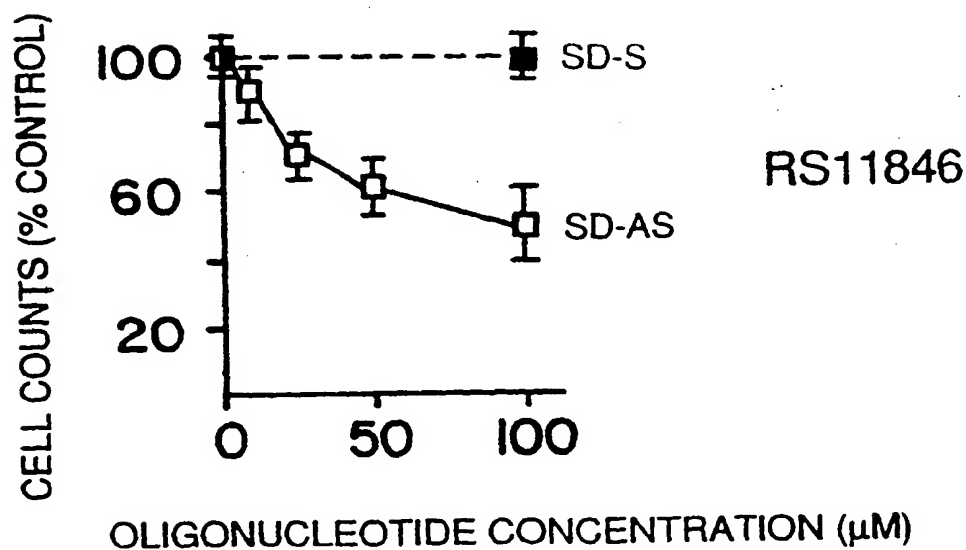


FIG. 1(b)





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FIG. 1(c)

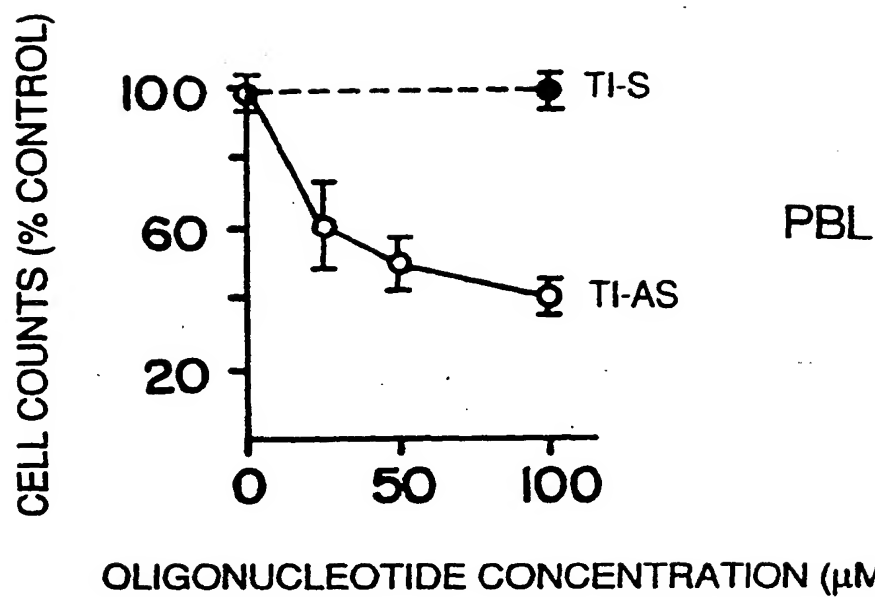
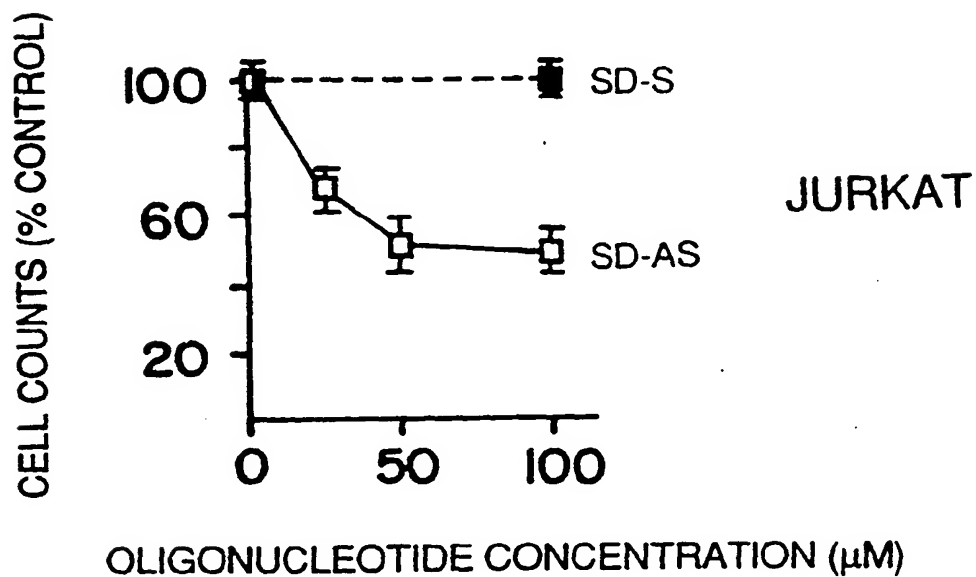


FIG. 1(d)



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FIG. 2(a)

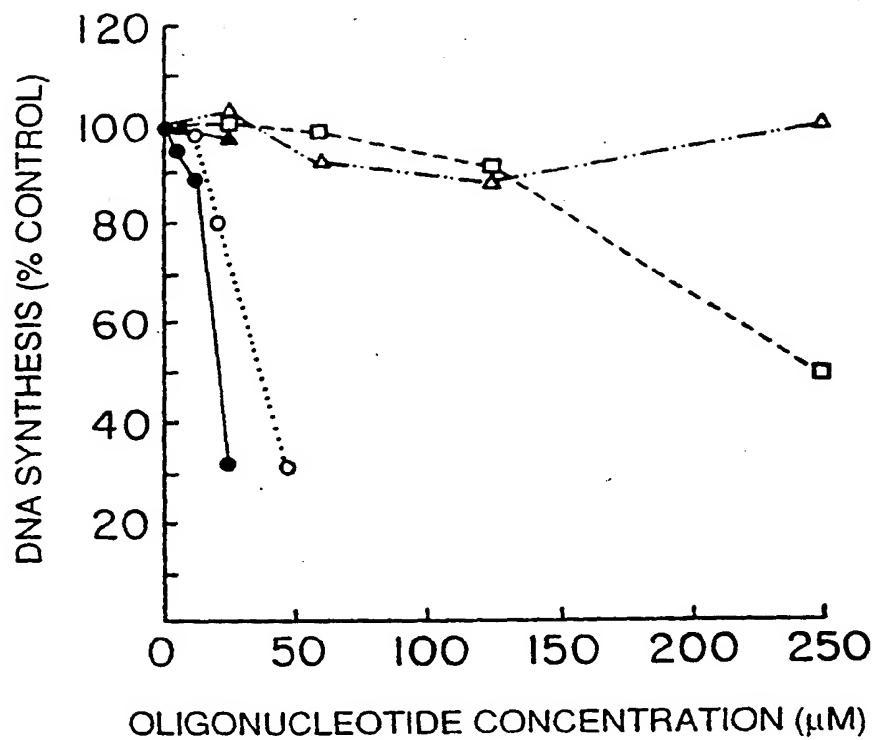
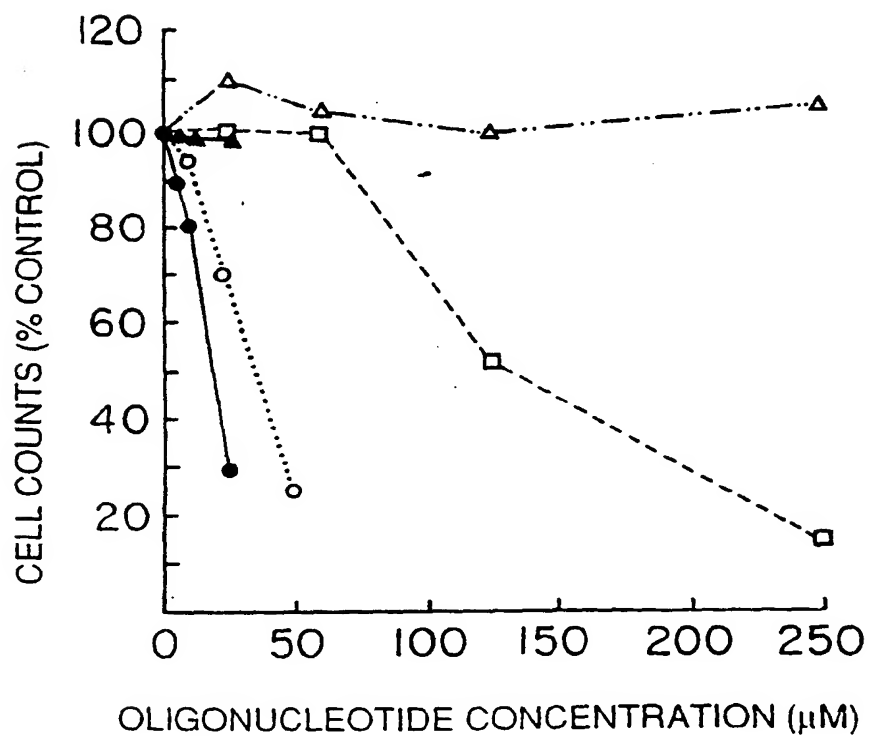


FIG. 2(b)



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01190-2  
C-01190 2  
01190-1  
01190-3  
01190-4  
01190-5  
01190-6  
U

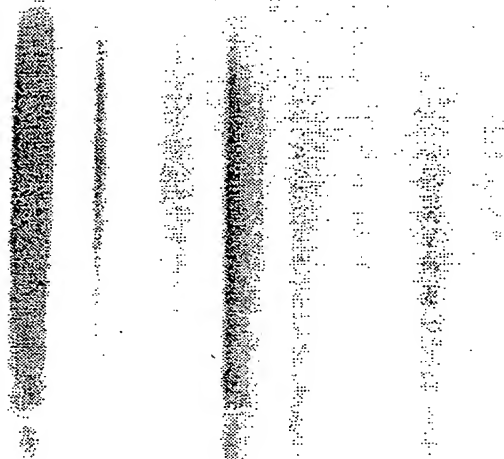


FIG. 3

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TI-AS  
TI-S  
SC 20  
U  
01190 2  
C-01190  
01190 3

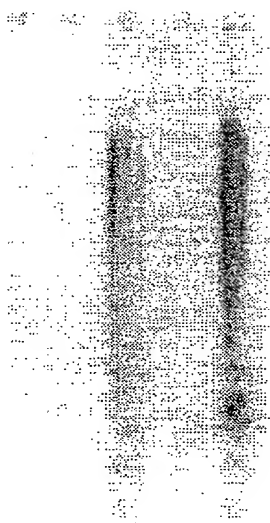


FIG. 4(a)

1432  
1406  
1394  
C-01190  
U

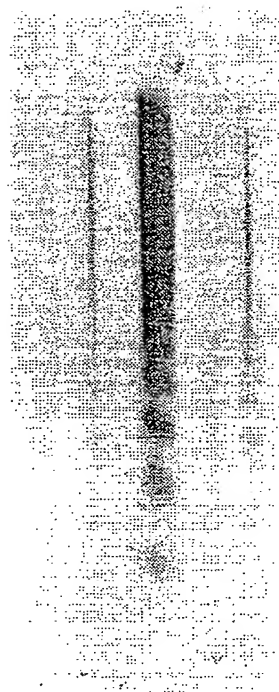
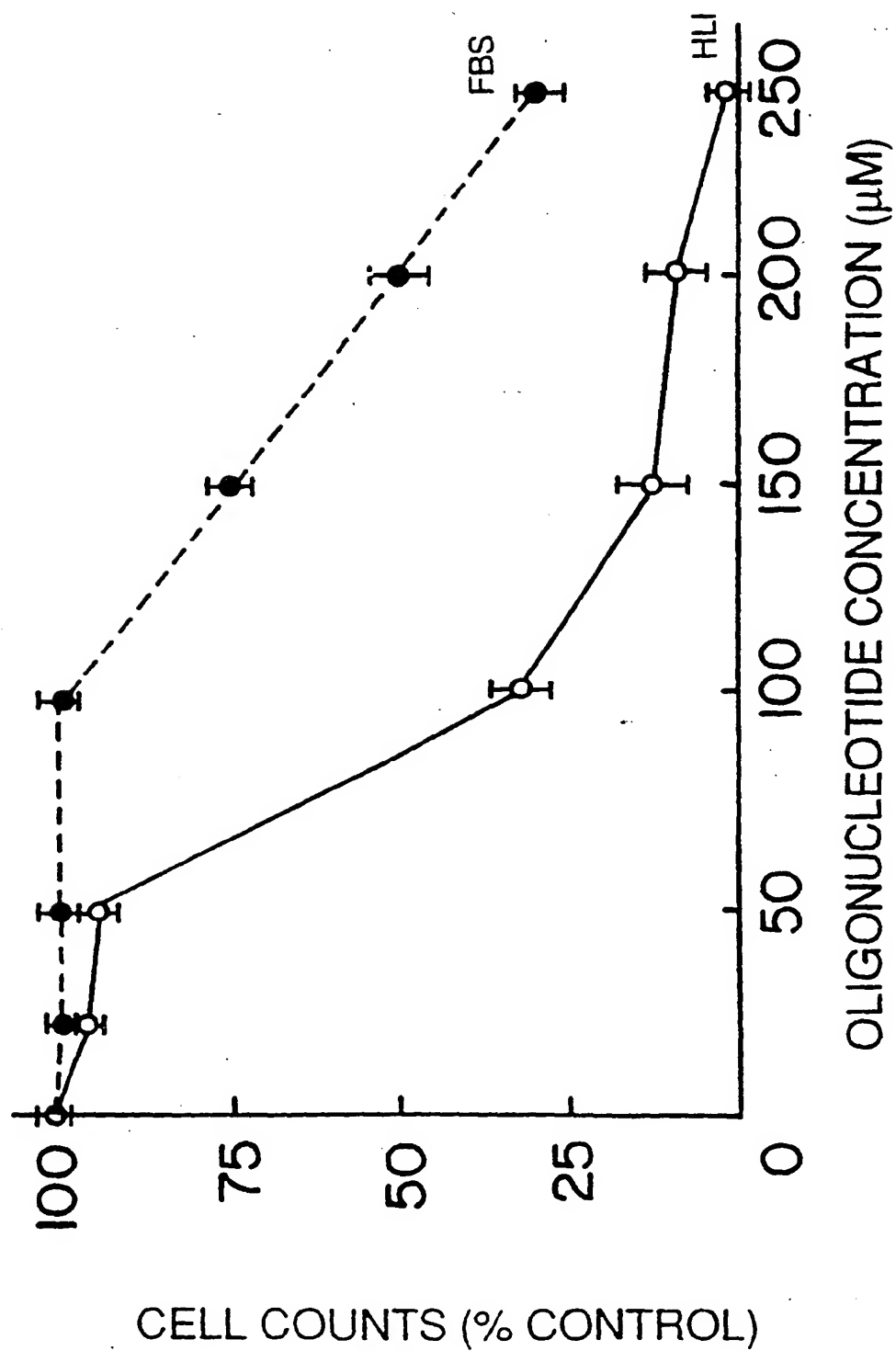


FIG. 4(b)

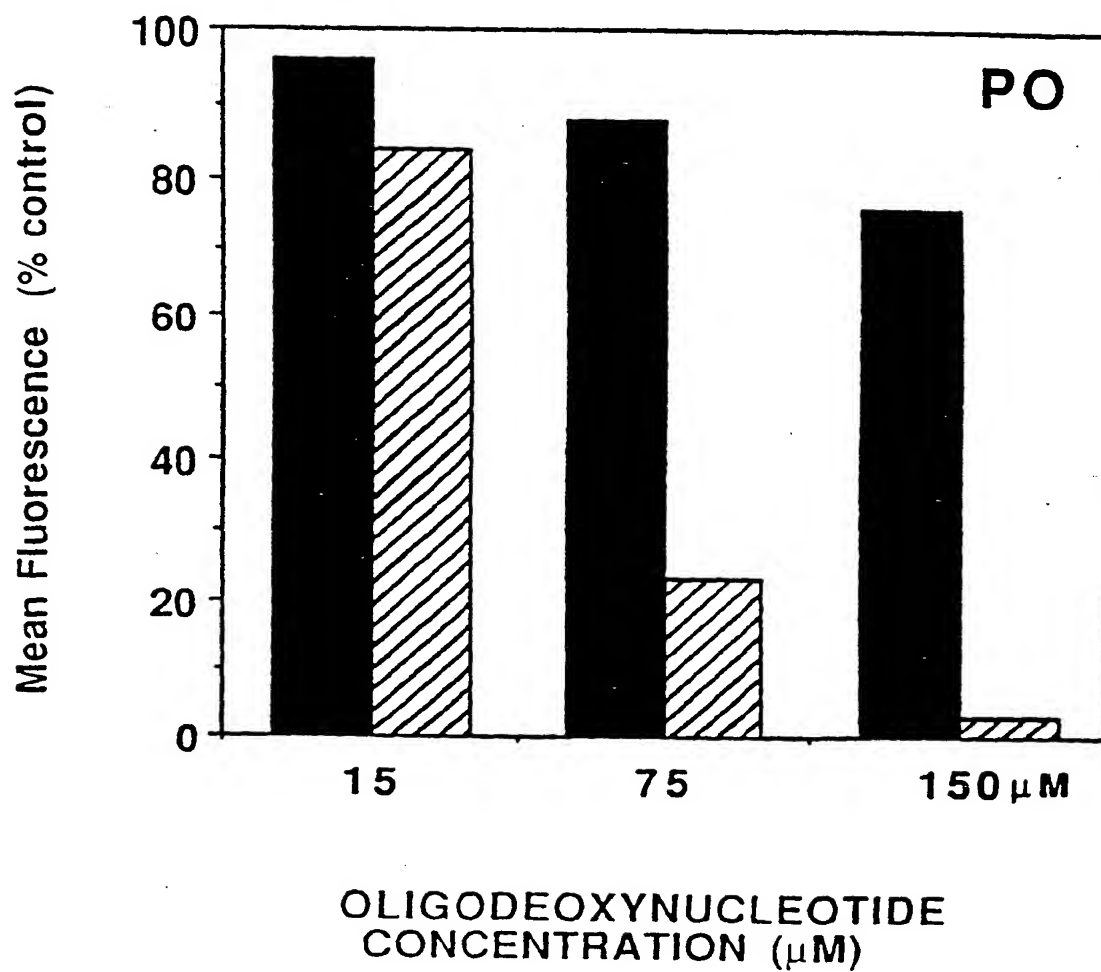
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FIG. 5



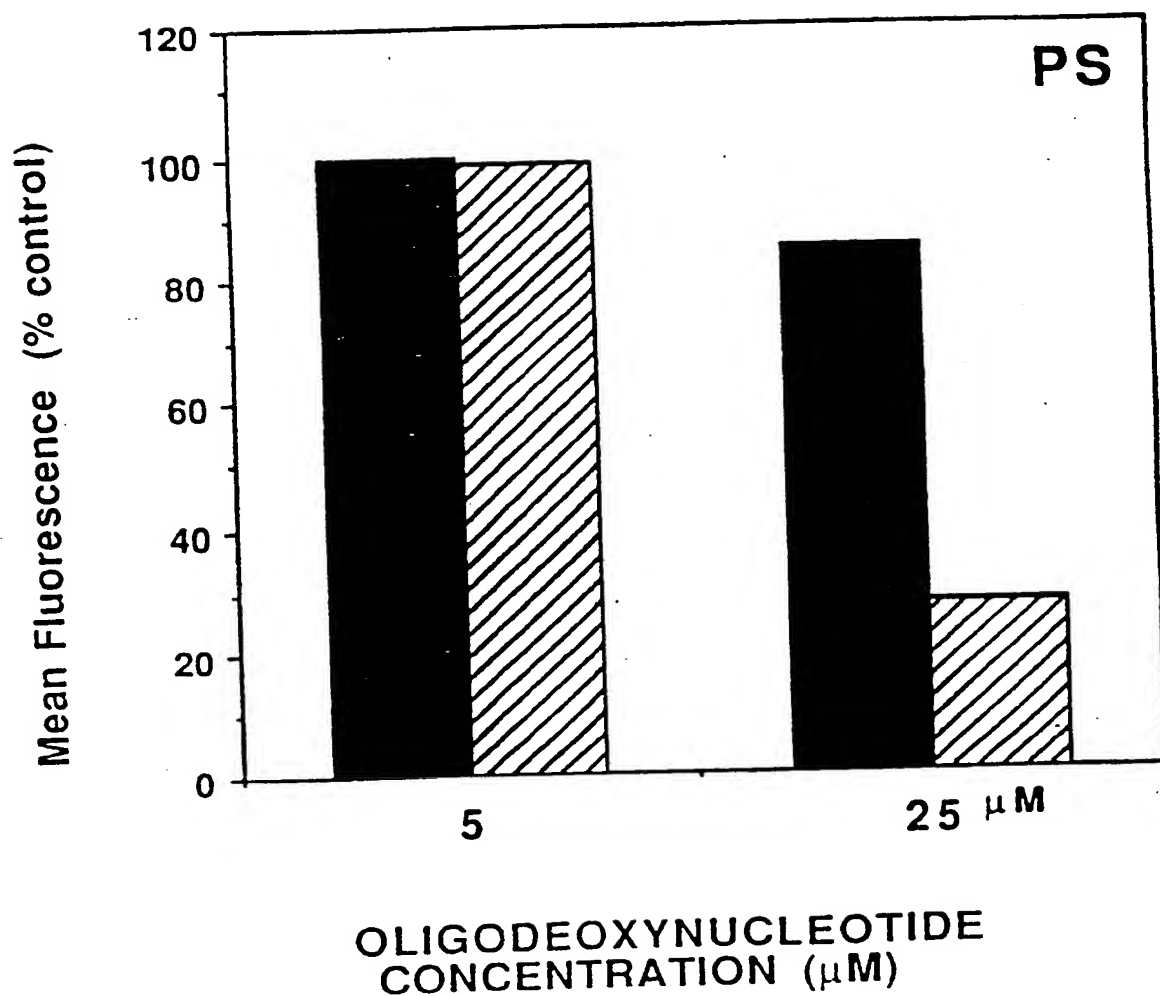
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FIG. 6(a)



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FIG. 6(b)



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FIG. 7(a)

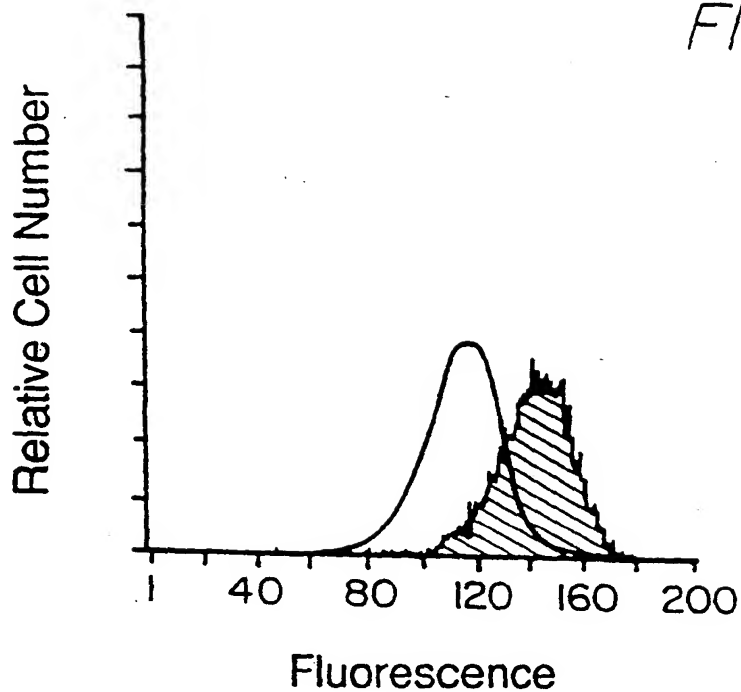
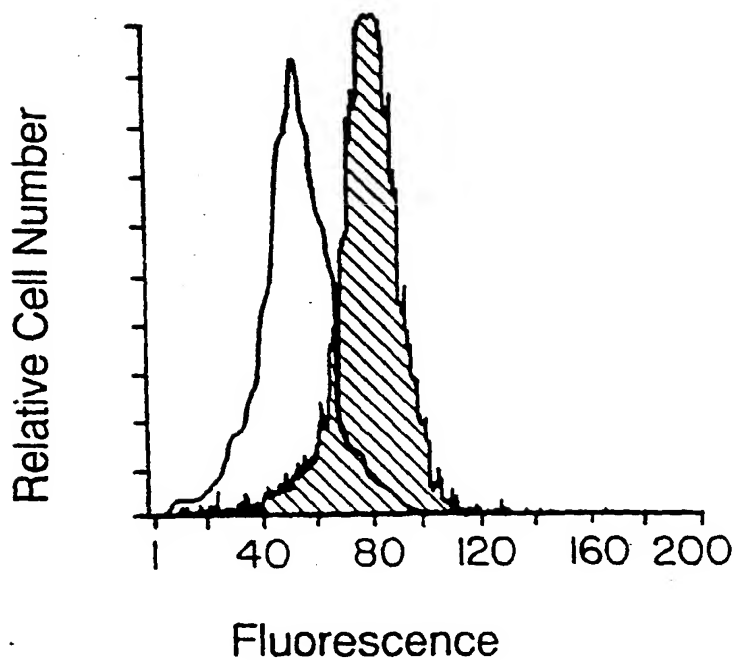


FIG. 7(b)





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FIG. 7(c)

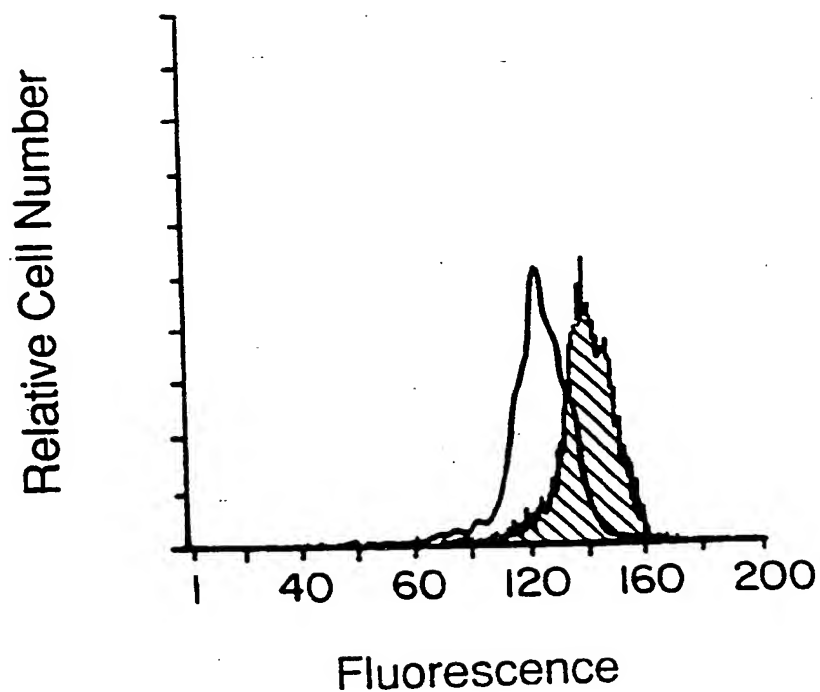
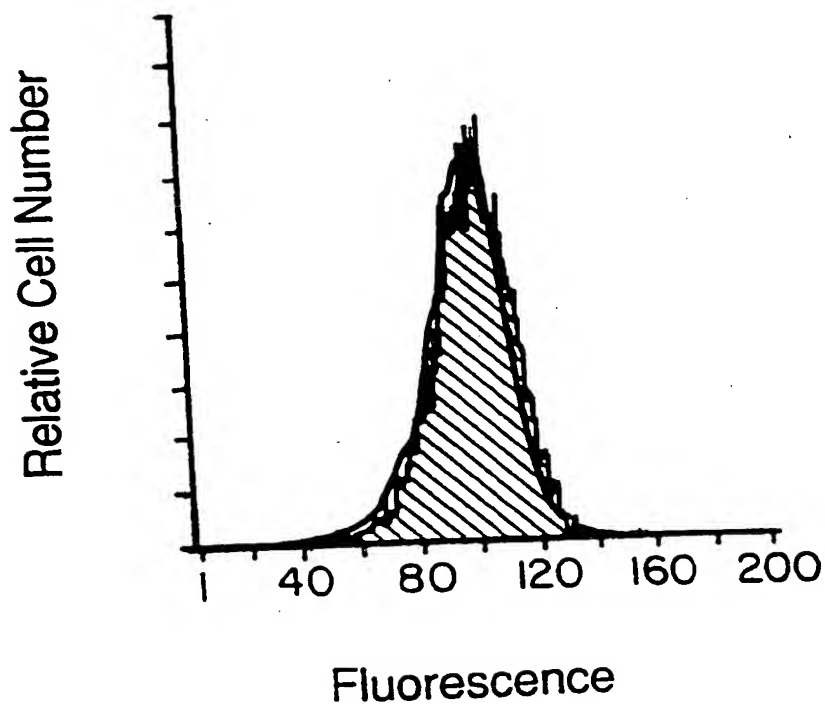


FIG. 7(d)



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FIG. 8A

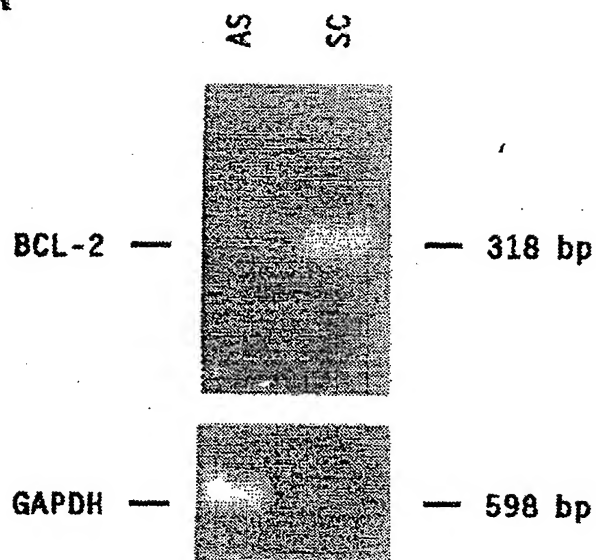
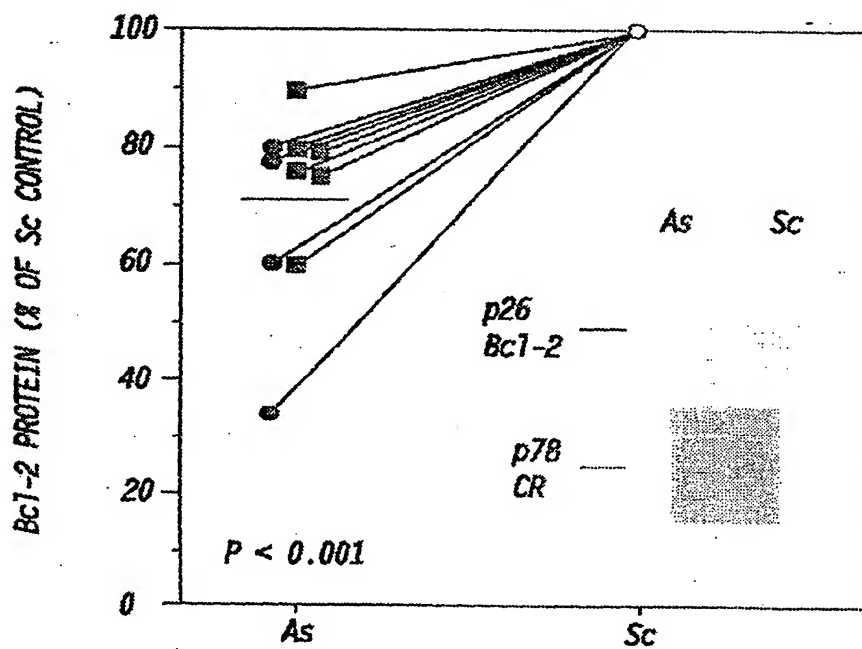
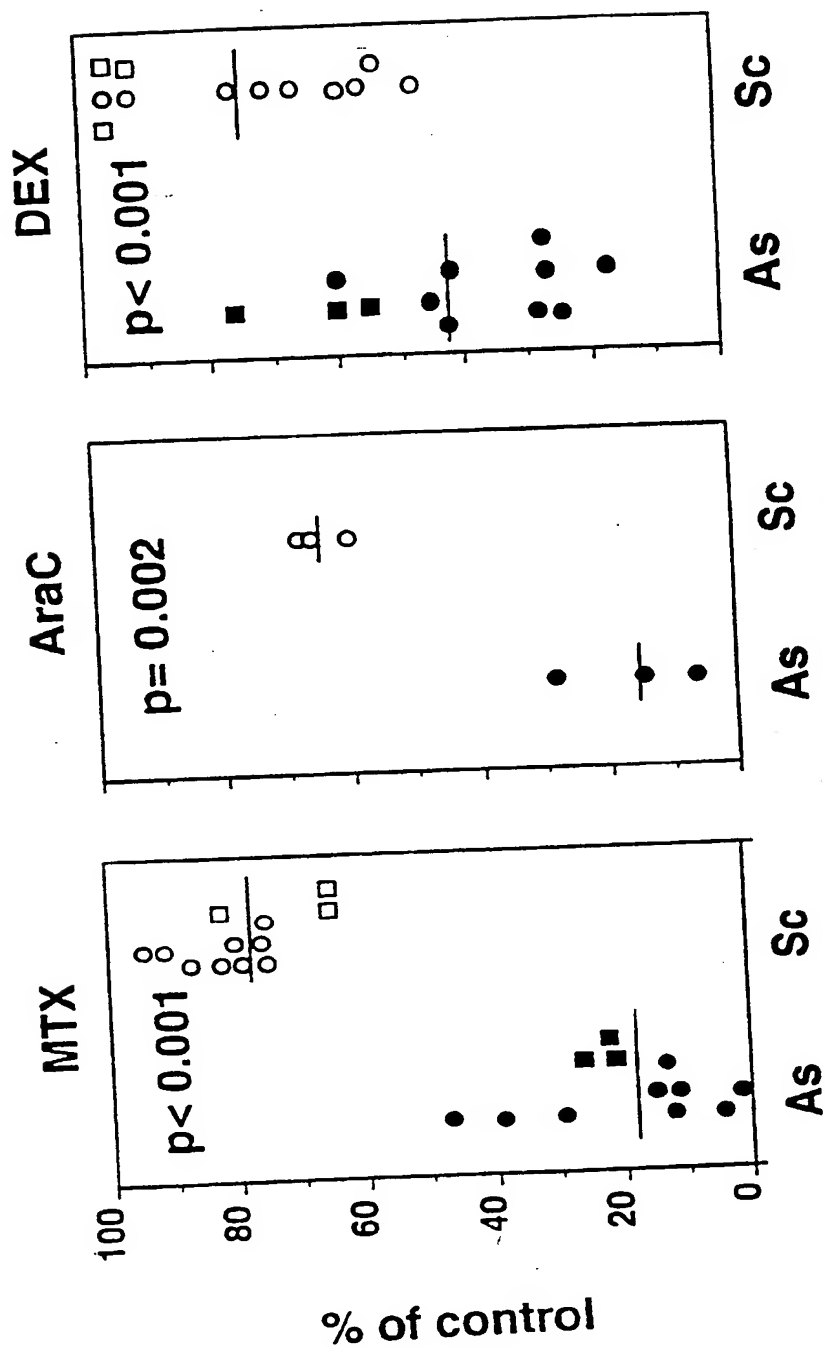


FIG. 8B



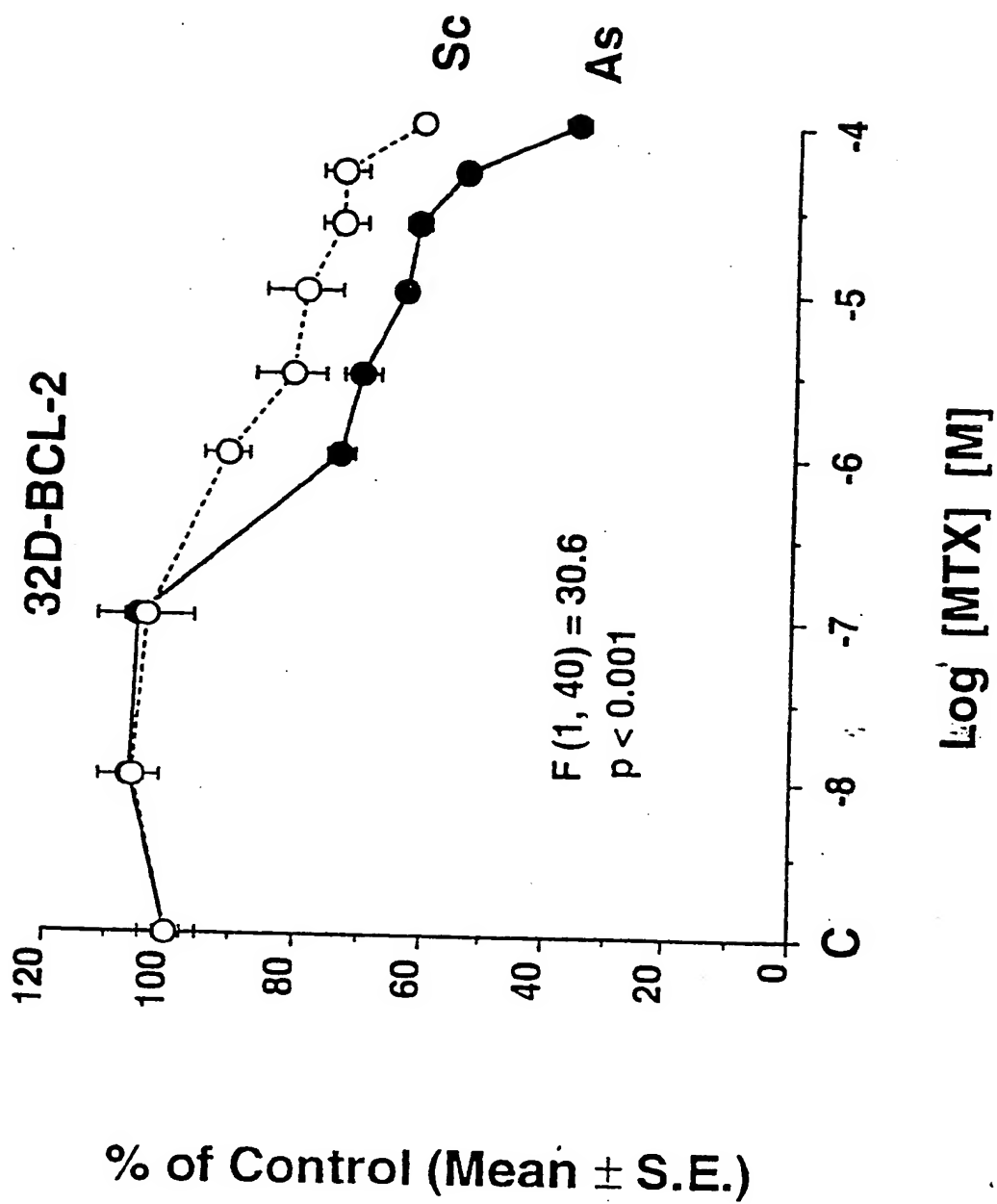
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FIG. 8C



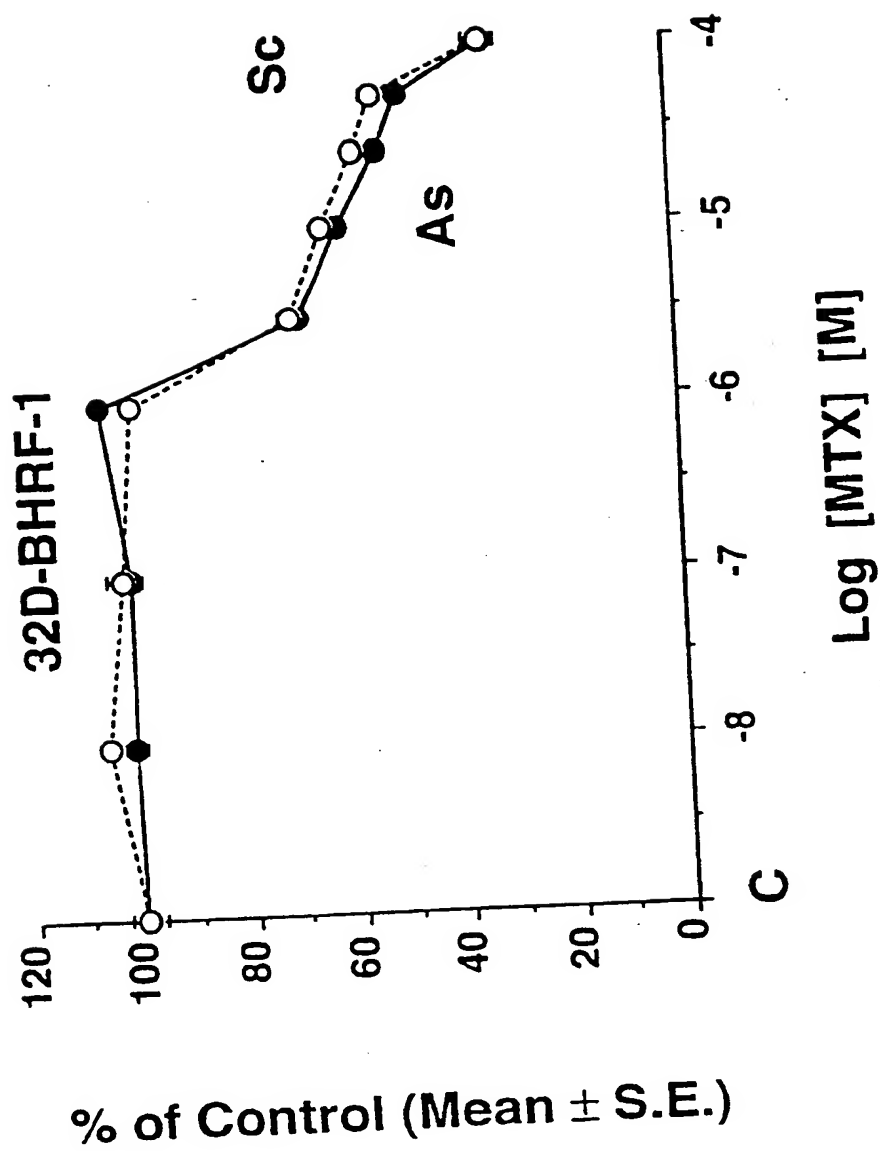
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FIG. 9(a)



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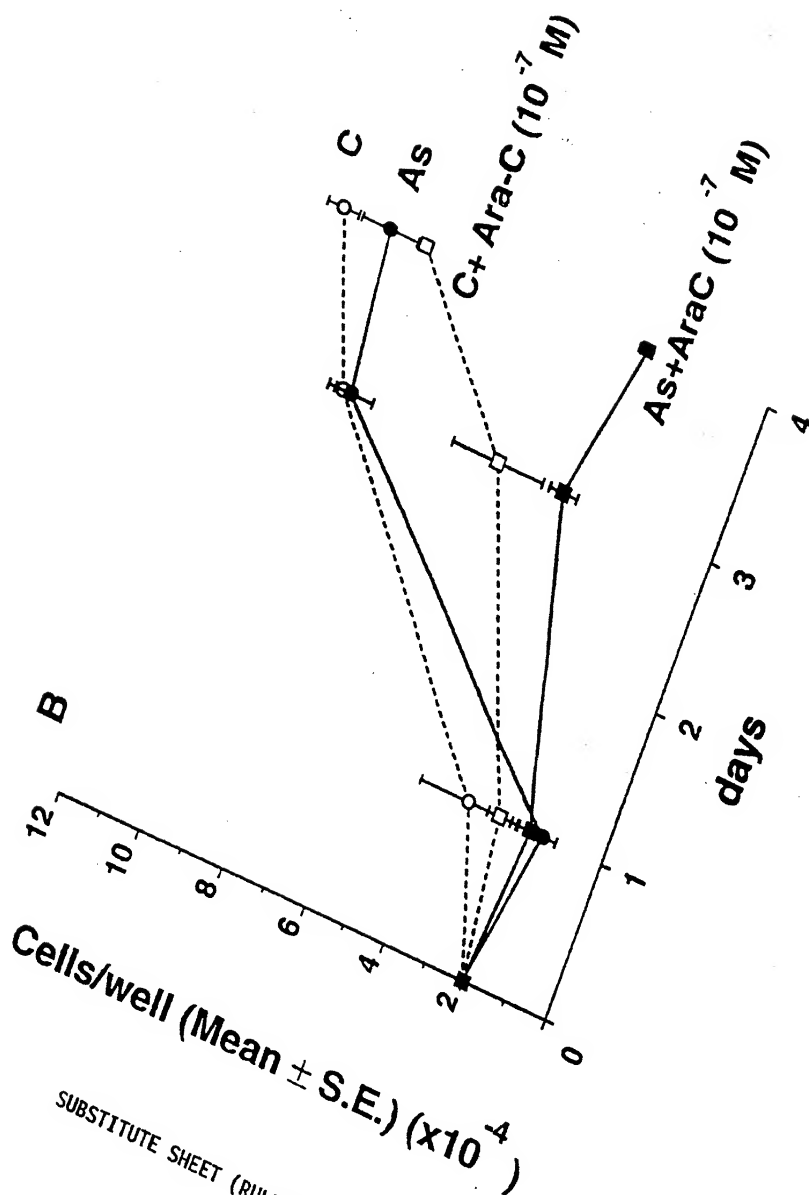
FIG. 9(b)



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PCT/US94/10725

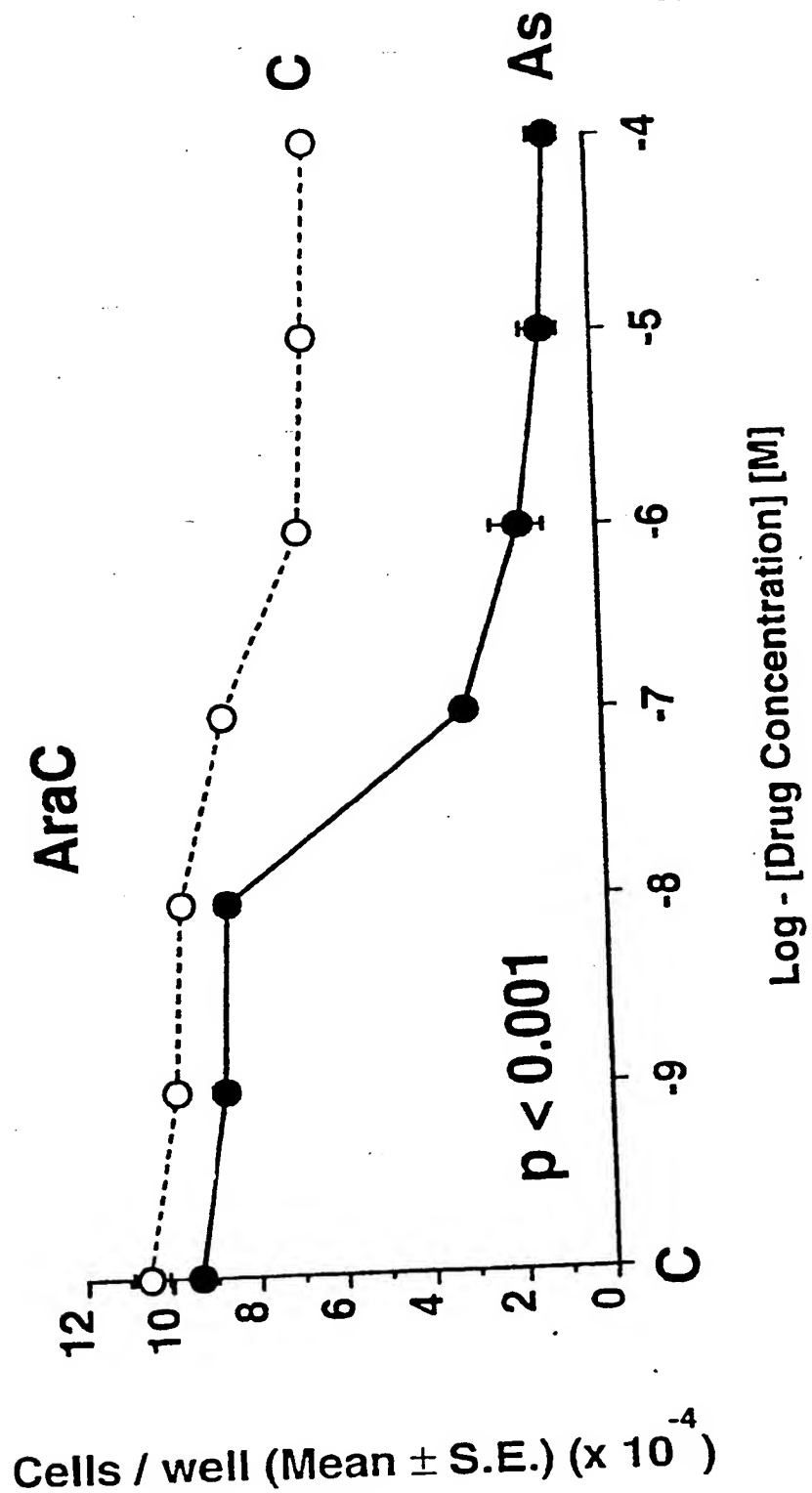
FIG. 10A



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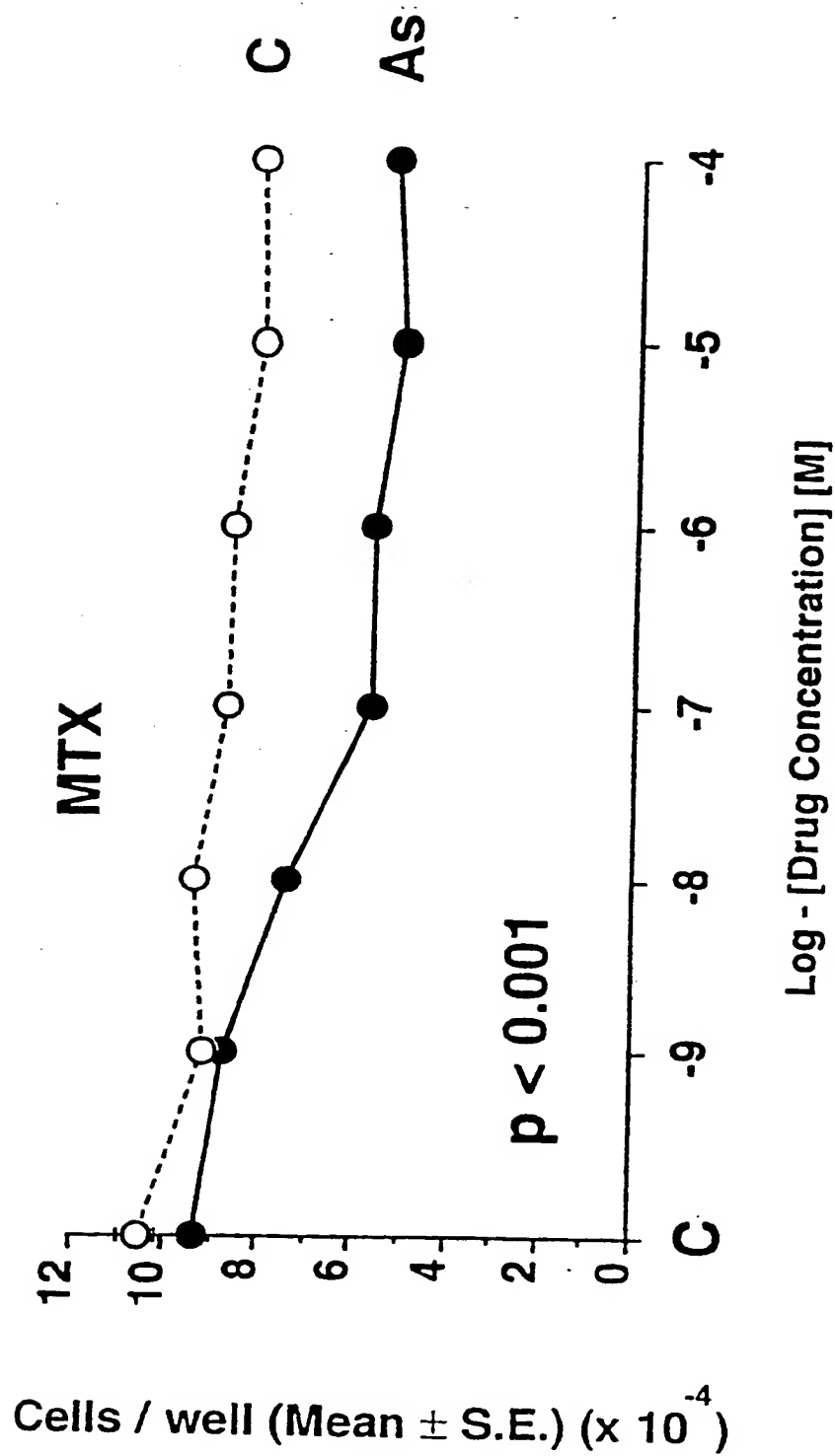
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FIG. 10B(1)



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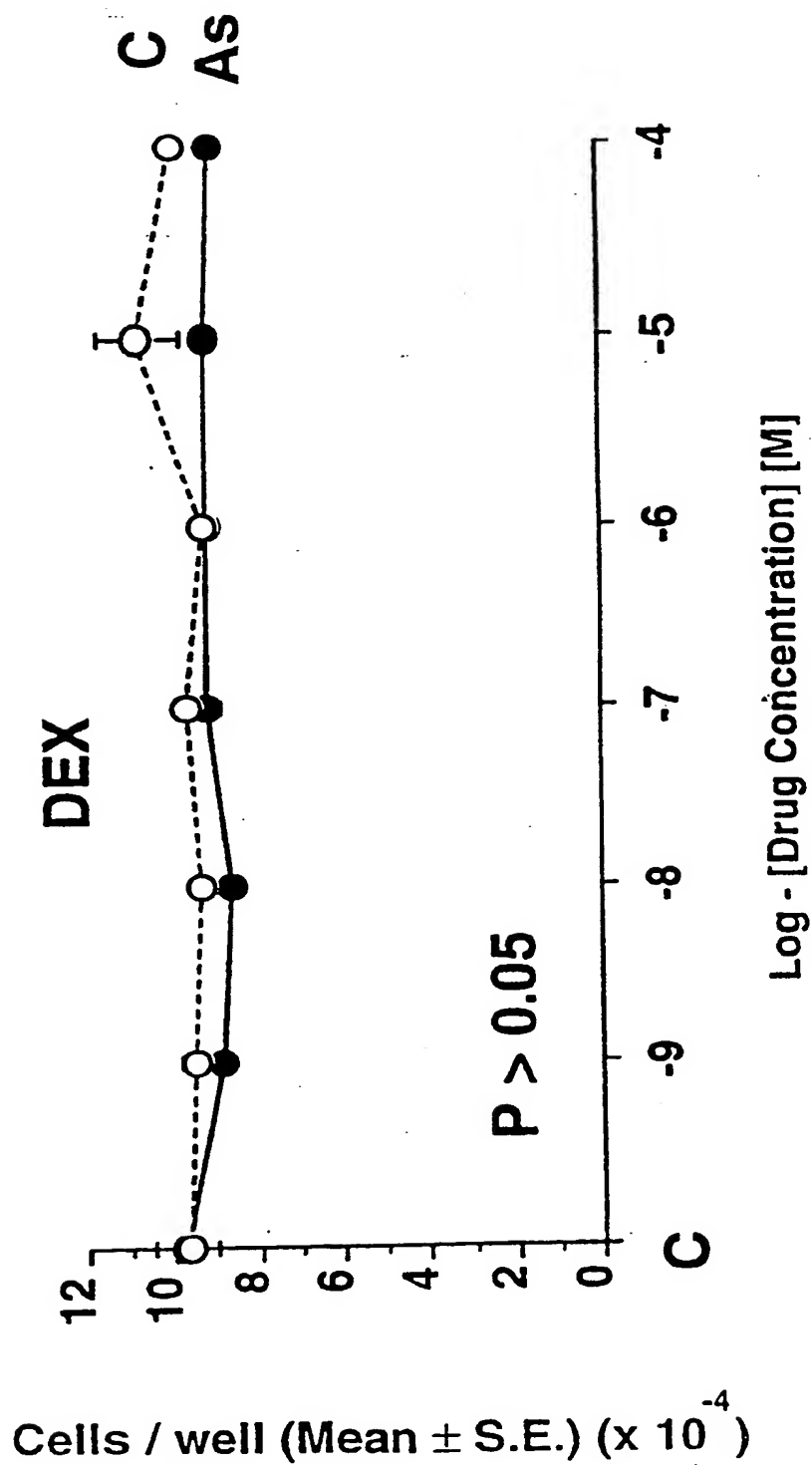
FIG. 10B(2)





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FIG. 10B (3)



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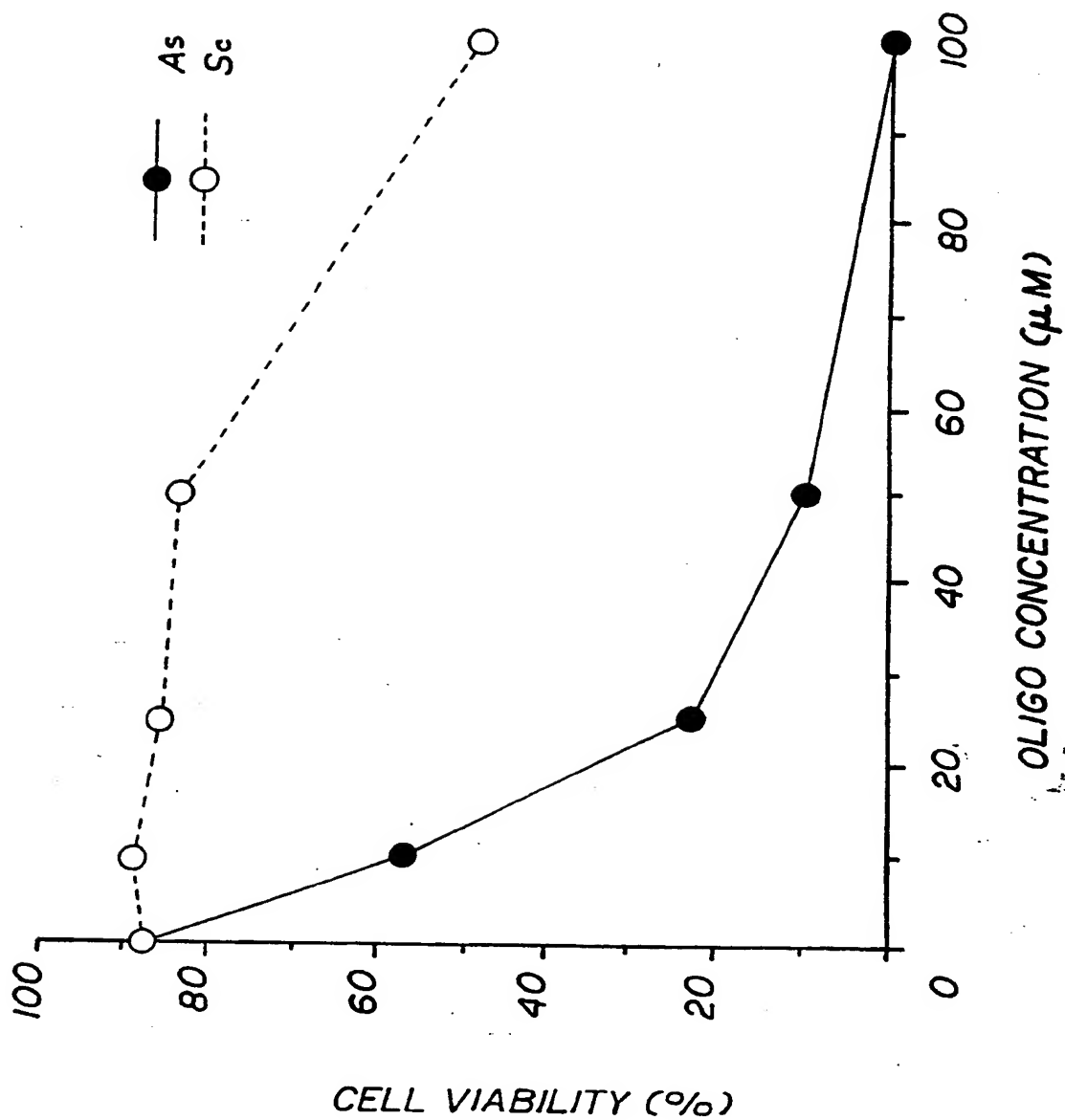


FIG. 11

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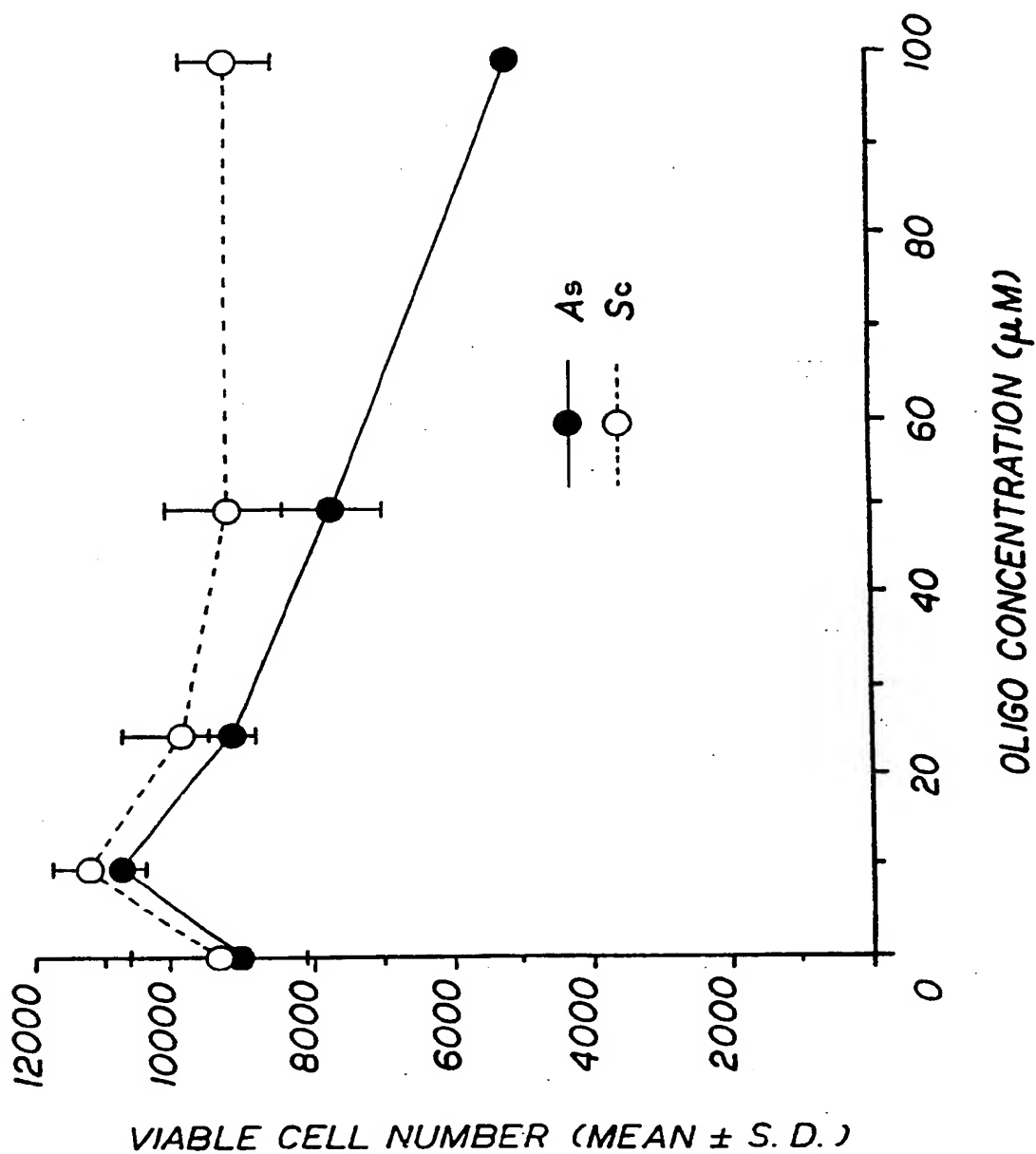
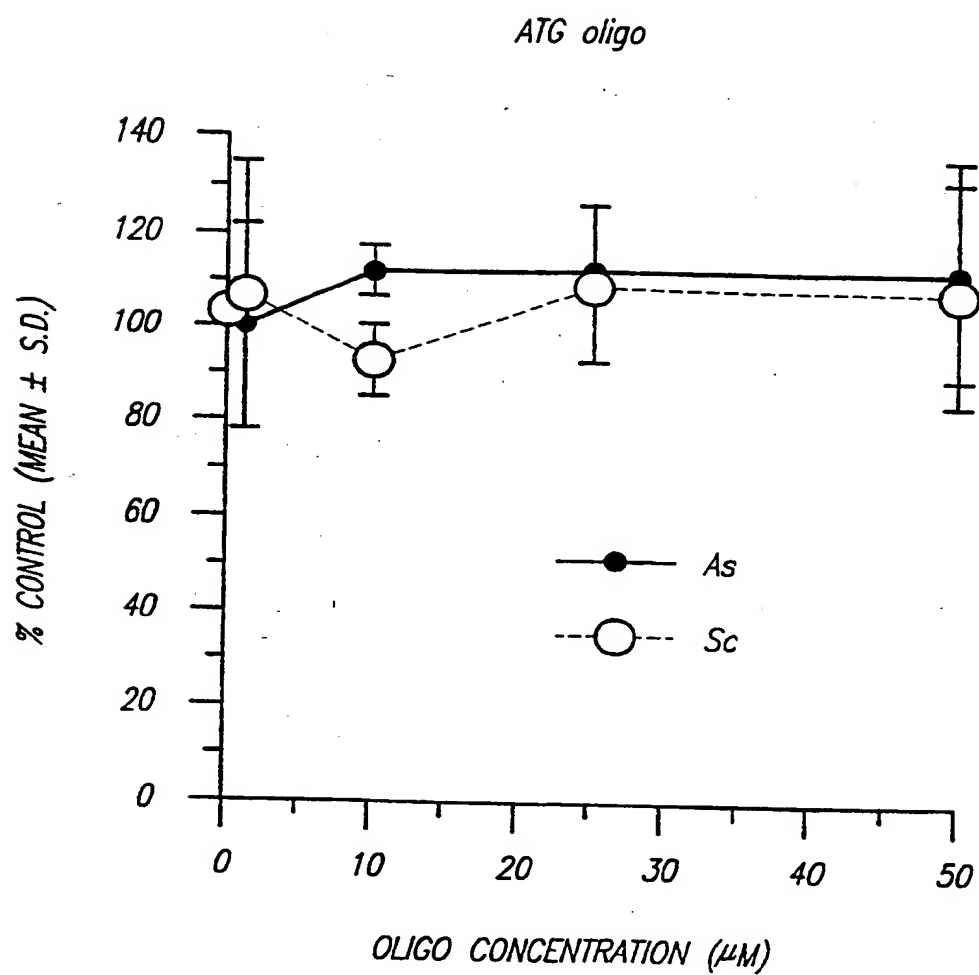


FIG. 12

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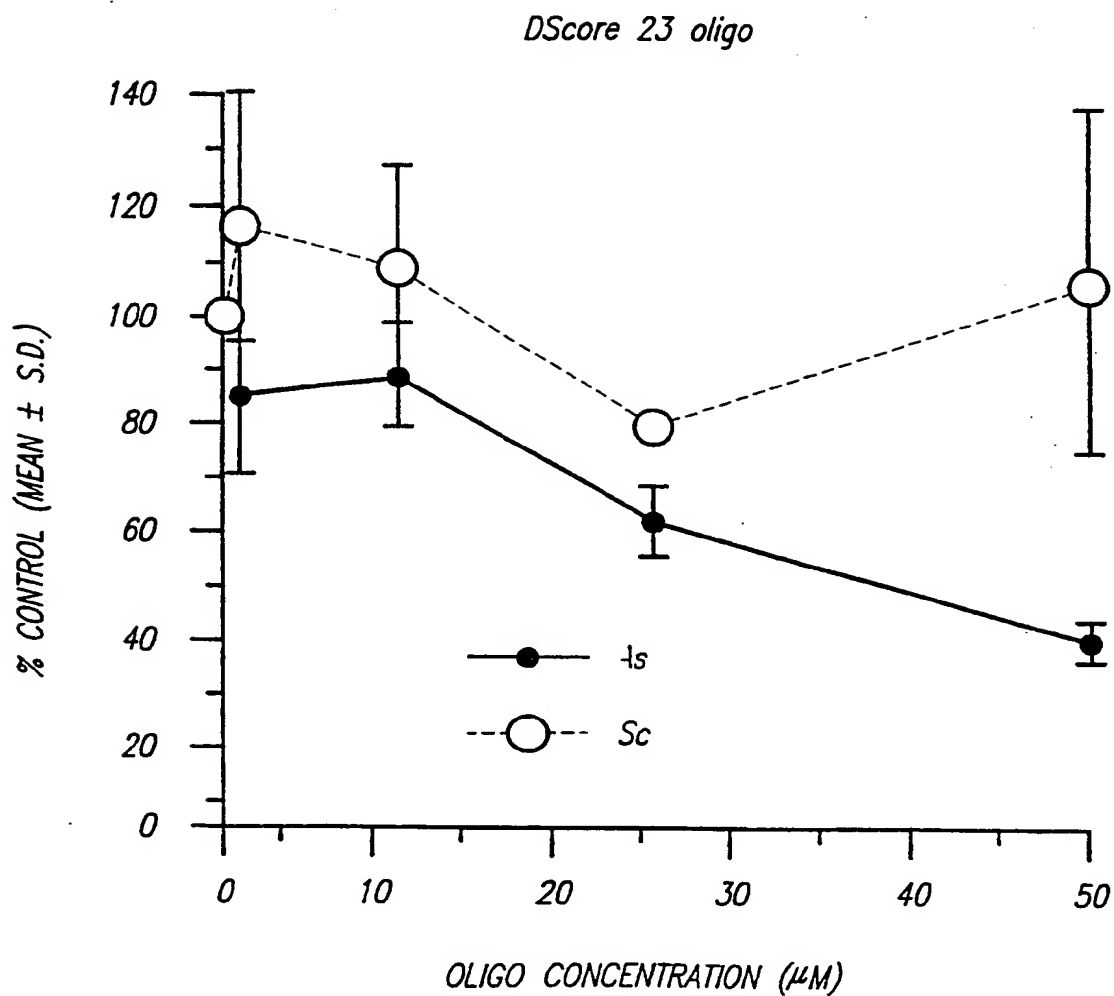
FIG. 13(a)



As-18' mer  
5'-TCTCCAGCGTGCGCCAT-3'  
Sc-18' mer  
5'-TGCACTCACGCTCGGCCT-3'

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FIG. 13(b)

*As23*

5'-GCCGCGGCGGGCGGGCGGGCA-3'

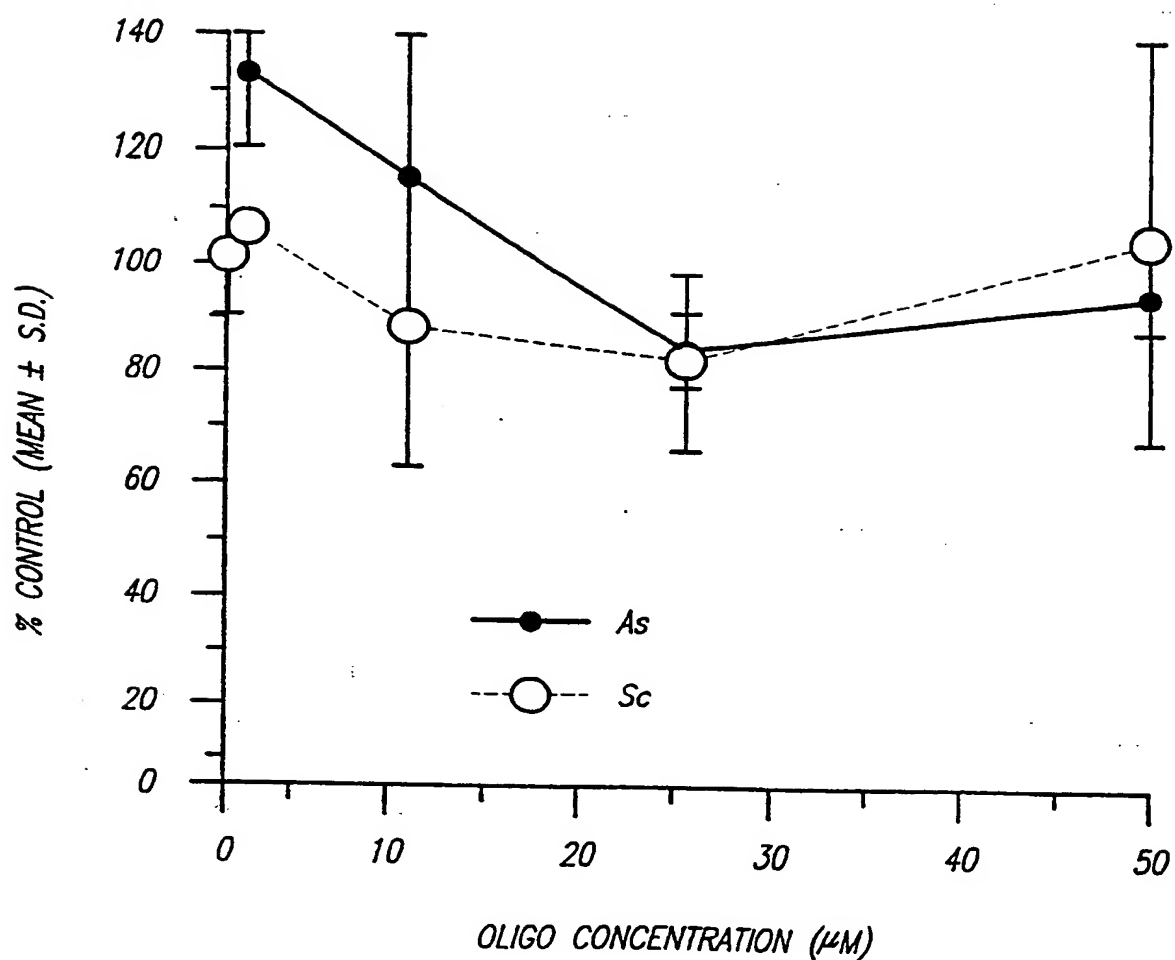
*Sc23*

5'-GGGCGGAGGCCGCGCGGCGG-3'

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FIG. 13(c)

DScore 72



As72  
5'-AGCGCGGCGGCGGCAGCGC-3'  
Sc72  
5'-GGGCCGGGAAGGGCGCCCCG-3'

## INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US 91/0725**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 48/00

US CL :514/44

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, bcl-2, antisense, anticode, apoptosis, tumor cells, therapeutic agent

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Anti-Cancer Drug Design, Volume 2, issued 1988, Paoletti, "Anti-sense oligonucleotides as potential antitumour agents: prospective views and preliminary results", pages 325-331, see entire article.	1-40
Y	Proceedings of the National Academy of Sciences USA, Volume 83, issued July 1986, Tsujimoto et al., "Analysis of the structure, transcripts and protein products of <i>bcl-2</i> , the gene involved in human follicular lymphoma", pages 5214-5218, see entire article.	1-40
Y	Cancer Research, Volume 48, issued 15 May 1988, Stein et al., "Oligodeoxynucleotides as inhibitors of gene expression: A review", pages 2659-2668, see entire article.	1-40

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

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Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

SUZANNE ZISKA, PH.D.

Telephone No. (703) 308-0196

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/10725

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Categ ry*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cancer Research, Volume 50, issued 15 October 1990, Reed et al., "Antisense-mediated inhibition of <i>BCL2</i> protooncogene expression and leukemic cell growth and survival: Comparisons of phosphodiester and phosphorothioate oligodeoxynucleotides", pages 6565-6570, see entire article.	1-40
Y	Cell, Volume 67, issued 29 November 1991, Strasser et al., " <i>bcl-2</i> transgene inhibits T cell death and perturbs thymic self-censorship", pages 889-899, see entire article.	1-40
Y	Chemical Reviews, Volume 90, No. 4, issued June 1990, Uhlmann et al., "Antisense oligonucleotides: A new therapeutic principle", pages 544-584, see entire article.	1-40
Y	US, A, 5,098,890 (GEWIRTZ ET AL.) 24 March 1992, see entire document.	1-40

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